

Neurotoxic effects on cerebellar granule cells and induction of ROS formation in human neutrophil granulocytes after exposure to polychlorinated biphenyls and penitrem A

Hanne Friis Berntsen



Master thesis in toxicology

Department of Biology

UNIVERSITY OF OSLO

November 2010

Acknowledgements

The work for this thesis was carried out at the Department of Biochemistry, Institute of Basic Medical Sciences, Faculty of Medicine, University of Oslo.

The PCB experiments were carried out as part of a bigger EU funded project, ATHON (Assessing the Toxicity and Hazard of Non-dioxin-like PCBs present in Food), that was aimed at studying the effects of non-coplanar PCBs.

The studies on penitrem A were part of a cooperation project between the Section of Chemistry and Toxicology at the National Veterinary Institute and the Institute of Basic Medical Sciences, Faculty of Medicine, University of Oslo.

I would like to thank my supervisor, Professor emeritus Frode Fonnum, for his dedication and support both during the experimental work and the last few months of writing up the thesis.

I would also like to thank Inger Lise Bogen for introducing me to work at the laboratory, experimental planning, and for always being available for questions, as well as reading through my thesis. Furthermore, I would like to thank my internal supervisor Professor Steinar Øvrebø for being available for questions, and Professor Sven Ivar Walaas for reading through my thesis, and for helpful discussions. I thank PhD student Angel Moldes-Anaya for great help with the issues related to the penitrem A part of my thesis.

I would like to thank Kine, Marit, Bjørg and Mattis in the scientific group for their help and support, and Professor Anne Karine Østvold and Kirsten Grundt for their help at the earlier phase of the experiments. I would also like to thank Professor Ketil Hylland for helping with statistics, and Kai for helping with the illustrations.

I thank Rita Tansø and Avi Ring at the Norwegian Defence Establishment for teaching me how to cultivate the cerebellar granule cells, and for helpful advice when I had problems with the cell cultures. I would also like to thank Stine Martinsen for helping with blood samples.

I would like to thank my boyfriend Christoffer and my family for understanding and support.

And last but not least, I thank my dear brother Christopher for sacrificing endless hours helping me with everything from statistics to proofreading, despite having his final exams in medicine only weeks away.

Hanne Friis Berntsen

Oslo, November 2010

Contents

ACKNOWLEDGEMENTS.....	2
SUMMARY	8
ABBREVIATIONS	10
1. INTRODUCTION AND BACKGROUND.....	12
1.1 ENVIRONMENTAL AND FOOD-BORNE TOXINS.....	12
1.2 POLYCHLORINATED BIPHENYLS.....	12
1.3 PENITREM A.....	15
1.4 REACTIVE OXYGEN SPECIES AND OXIDATIVE STRESS	17
1.4.1 Formation of reactive oxygen species.....	17
1.4.2 Antioxidant systems and oxidative stress	20
1.4.3 Oxidative stress in the brain.....	21
1.5 CEREBELLAR GRANULE CELLS.....	22
1.5.1 Cerebellar granule cells as a model.....	22
1.5.2 The MTT assay for detection of cell death	23
1.6 HUMAN NEUTROPHIL GRANULOCYTES.....	24
1.6.1 ROS formation in human neutrophil granulocytes and choice of model	24
1.6.2 Detection of reactive oxygen species in vitro	25
1.6.3 Lactate dehydrogenase assay.....	26
1.7 CYTOPROTECTIVE AGENTS AND INHIBITORS OF ROS PRODUCTION.....	26
1.8 AIM OF STUDY	29
2. MATERIALS AND METHODS	30

2.1	CHEMICALS	30
2.1.1	<i>Chemicals for the cultivation of cerebellar granule cells and the MTT assay:</i>	<i>30</i>
2.1.2	<i>Chemicals for the isolation and experiments with human neutrophil granulocytes: ..</i>	<i>31</i>
2.1.3	<i>Test chemicals.....</i>	<i>31</i>
2.2	LABORATORY ANIMALS.....	34
2.3	CEREBELLAR GRANULE CELL EXPERIMENTS.....	35
2.3.1	<i>Cultivation of cerebellar granule cells</i>	<i>35</i>
2.3.2	<i>In vitro toxin exposure of cerebellar granule cells</i>	<i>36</i>
2.3.3	<i>MTT cell survival assay</i>	<i>39</i>
2.4	HUMAN NEUTROPHIL GRANULOCYTE EXPERIMENTS.....	40
2.4.1	<i>Isolation of human neutrophil granulocytes</i>	<i>40</i>
2.4.2	<i>Measurement of reactive oxygen species after exposure to toxic substances</i>	<i>41</i>
2.4.3	<i>Detection of ROS with the DCF assay</i>	<i>42</i>
2.4.4	<i>Detection of ROS with the luminol chemiluminescence assay.....</i>	<i>43</i>
2.4.5	<i>LDH assay.....</i>	<i>43</i>
2.5	STATISTICAL ANALYSES	44
3.	RESULTS.....	48
3.1	NEUROTOXICITY STUDIES IN CEREBELLAR GRANULE CELLS	48
3.1.1	<i>General considerations.....</i>	<i>48</i>
3.1.2	<i>Comparison of PCB 52 and PCB 180.....</i>	<i>48</i>
3.1.3	<i>Effects of potentially neuroprotective substances on PCB 52 and PCB 180 toxicity..</i>	<i>50</i>
3.1.4	<i>Neurotoxicity of low and highly chlorinated PCBs.....</i>	<i>53</i>
3.1.5	<i>Neurotoxicity of penitrem A at different concentrations</i>	<i>55</i>
3.1.6	<i>Penitrem A tested with potentially neuroprotective substances</i>	<i>56</i>

3.1.7	<i>Survival after different exposure times to penitrem A and penitrem A with vitamin E</i>	60
3.2	ROS FORMATION IN HUMAN NEUTROPHIL GRANULOCYTES	61
3.2.1	<i>ROS formation after PCB exposure measured with the DCF assay.....</i>	61
3.2.2	<i>DCF assay measurements of ROS production after PCB exposure in conjunction with potential ROS inhibitors</i>	63
3.2.3	<i>Luminol-chemiluminescence assay measurements of ROS production after exposure to PCBs</i>	69
3.2.4	<i>Luminol assay measurements of ROS production after exposure to PCBs in conjunction with ROS inhibitors</i>	70
3.2.5	<i>Preliminary studies of penitrem A-induced ROS production</i>	75
3.2.6	<i>Measurements of penitrem A-induced ROS production using the DCF assay.....</i>	77
3.2.7	<i>Potential inhibitors and their effect on ROS production after penitrem A exposure, assessed with the DCF-fluorescence assay</i>	78
3.3	LDH ASSAY.....	81
4.	DISCUSSION.....	82
4.1	MAIN RESULTS	82
4.2	CEREBELLAR GRANULE CELLS.....	82
4.2.1	<i>Cerebellar granule cells as a model.....</i>	82
4.2.2	<i>Toxicity studies of low and highly chlorinated PCBs.....</i>	83
4.2.3	<i>Potential mechanisms of PCB neurotoxicity</i>	85
4.2.4	<i>Penitrem A neurotoxicity.....</i>	88
4.2.5	<i>Possible mechanisms of penitrem A neurotoxicity</i>	88
4.2.6	<i>Neurotoxicity of penitrem A at different times of exposure</i>	91
4.3	HUMAN NEUTROPHIL GRANULOCYTES.....	92
4.3.1	<i>Human neutrophil granulocytes as a model.....</i>	92

4.3.2	<i>ROS production in human neutrophil granulocytes after PCB exposure</i>	<i>92</i>
4.3.3	<i>Assessment of pathways involved in ROS production after PCB exposure.....</i>	<i>94</i>
4.3.4	<i>ROS production in human neutrophil granulocytes after penitrem A exposure</i>	<i>97</i>
4.3.5	<i>Mechanisms of ROS-induction after penitrem A exposure</i>	<i>98</i>
5.	CONCLUSIONS AND FUTURE PERSPECTIVES	99
6.	REFERENCES	101
7.	APPENDIX	109
7.1	SOLUTIONS FOR CEREBELLAR GRANULE CELL EXPERIMENTS.....	109
7.1.1	<i>Solutions for the cultivation of cerebellar granule cells</i>	<i>109</i>
7.1.2	<i>Solutions for exposure of cells to toxic substances and MTT cell survival assay</i>	<i>110</i>
7.2	SOLUTIONS FOR HUMAN NEUTROPHIL GRANULOCYTE EXPERIMENTS.....	110
7.2.1	<i>Solutions for human neutrophil granulocyte experiments</i>	<i>110</i>

Summary

Polychlorinated biphenyls (PCBs) are persistent organic pollutants that bioaccumulate and biomagnify through the food chain. Exposure to *ortho*-chlorinated PCBs has been associated with effects on the nervous and immune systems. Penitrem A is a mycotoxin found to cause neurological symptoms in dogs after ingestion of mouldy food. There have also been cases of suspected penitrem A intoxication in humans.

The aim of this study was to investigate differences in neurotoxicity in cerebellar granule cells between low and highly chlorinated *ortho*-substituted PCBs *in vitro*, and mechanisms underlying their toxicity. The neurotoxic potential of penitrem A was also investigated. Furthermore, the ability of different PCB congeners and of penitrem A to induce ROS production was investigated in human neutrophil granulocytes *in vitro*.

Cerebellar granule cells were exposed to low and highly chlorinated PCBs, as well as to penitrem A. Effects of potentially neuroprotective substances was also assessed after exposure to the toxic substances. The MTT assay was used to assess cell survival.

Human neutrophil granulocytes were also exposed to low and highly chlorinated PCBs and penitrem A. ROS production after exposure was assessed with the DCF and luminol chemiluminescence assays. Effects of inhibitors of ROS production was assessed to identify potential pathways involved in the ROS production.

In this study, the higher chlorinated PCBs were more toxic to cerebellar granule cells *in vitro* than lower chlorinated congeners. Cytoprotective effects were found for the substances vitamin E and MK-801. This may indicate involvement of ROS production and NMDA receptors in PCB neurotoxicity. The lower chlorinated PCBs induced more ROS production in human neutrophil granulocytes than the higher chlorinated congeners. Co-incubation with the substances U0126, BAPTA-AM, vitamin E, SP600125, SB203580, FK-506 and CsA significantly reduced ROS levels.

The mycotoxin penitrem A caused more cell death in cerebellar granule cells with increasing concentration and exposure time, and increasing concentrations also induced a dose dependent increase in ROS production in human neutrophil granulocytes. Co-incubation with GABA, phenobarbital, diazepam, vitamin E, BAPTA-AM, SP600125, FK-506 and

cyclosporine A all reduced granule cell death. This may indicate that the GABA receptor, ROS production, disruption of calcium homeostasis and activation of the pro-apoptotic JNK pathway are involved in penitrem A neurotoxicity.

U0126, SP600125, SB203580, FK-506, cyclosporine A, vitamin E and BAPTA-AM all reduced ROS production in granulocytes after penitrem A exposure. This may point to an involvement of the MEK 1/2, MEK 5, p38 and JNK pathways in the mechanism of penitrem A-induced ROS production.

Abbreviations

Ah	Aryl hydrocarbon
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ARA-C	Cytosine β -D-arabinofuranoside
AUC	Area under the curve
BAPTA-AM	1,2-bis(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester
BK (channel)	High conductance calcium activated potassium channel
BME	Basal medium Eagle
BSA	Bovine serum albumine
cGMP	Cyclic guanosine monophosphate
CI	Confidence interval
CsA	Cyclosporine A
DAG	Diacylglycerol
DCHF	2',7'-dichlorodihydrofluorescein
DCHF-DA	2',7'-dichlorodihydrofluorescein diacetate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-signal-regulated kinase
FBS	Foetal bovine serum
FK-506	Tacrolimus
GABA	Gamma-aminobutyric acid
GSH	Reduced glutathione
GSH-Px	Glutathione peroxidase
GSSG	Oxidised glutathione
HBSS	Hanks' Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
IU	International units
JNK	c-Jun amino-terminal kinase
LAF	Laminar air flow
LC ₅₀	Lethal concentration 50

LDH	Lactate dehydrogenase
L-NAME	N- ω -nitro-L-arginine methyl ester
MAPK/MAP kinase	Mitogen activated protein kinase
MEK	Mitogen activated protein kinase kinase
MK-801	(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate
MPO	Myeloperoxidase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NAD	Nicotine amide adenine dinucleotide
NADH	Reduced nicotine amide adenine dinucleotide
NADPH	Reduced nicotine-amide adenine dinucleotide phosphate
NBQX	2,3-dihydroxo-6-nitro-7-sulfamoyl-benzo(F)quinoxaline
NF	Nuclear factor
NMDA	N-methyl-D-aspartate
NOS	Nitric oxide synthase
NVI	National Veterinary Institute
PA	Phosphatidic acid
PCB	Polychlorinated biphenyl
PKC	Protein kinase C
PLA ₂	Phospholipase A ₂
PLC	Phospholipase C
PLD	Phospholipase D
RAF	RAF gene
RAS	RAt Sarcoma (gene)
ROS	Reactive oxygen species
SB203580	4-[4-fluorophenyl]-2-[4-methylsulfinylphenyl]-5-[4-pyridyl]-1H-imidazole
SE	Standard error of the mean
SOD	Superoxide dismutase
SP600125	Anthra[1,9-cd]pyrazol-6(2H)-one
TLC	Thin layer chromatography
TRK	Tyrosine kinase

1. Introduction and background

1.1 Environmental and food-borne toxins

Several toxic substances that are present in food have the potential to affect the central nervous system in man after ingestion. Among these are the polychlorinated biphenyls (PCBs), which are environmental toxicants that have been found to bioaccumulate and biomagnify through the food chain. Humans may be exposed through foodstuffs such as fatty fish, shellfish, meat, liver, eggs and dairy products (La Rocca and Mantovani, 2006).

PCBs have been associated with hyperactivity and impaired cognitive functions in children after exposure via mother's milk, and decreased motor activity in exposed adults (Mariussen and Fonnum, 2006). Immunotoxic effects have also been reported, including altered gammaglobuline levels in human blood after PCB intoxication (Nakanishi *et al.*, 1985), altered T- and NK cell function (Safe, 1994) as well as effects on neutrophil granulocytes (Ganey *et al.*, 1993; Voie *et al.*, 1998). Thus, it is of interest to elucidate mechanisms of PCB toxicity. For this thesis, *in vitro* models using cerebellar granule cells and human neutrophil granulocytes were chosen for studies of cell death and production of reactive oxygen species (ROS), respectively.

During the work on the thesis, the cell models used were found to be suitable to study neurotoxic effects of another toxic substance found in food, which has also been associated with altered neurological function. The mycotoxin penitrem A has been shown to cause tremor, ataxia and convulsions in dogs (Moldes-Anaya *et al.*, 2010), and there have also been reports of suspected penitrem A intoxications in man causing neurological symptoms (Cole *et al.*, 1983; Lewis *et al.* 2005).

1.2 Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) are examples of synthetic persistent organic pollutants that are found widespread in nature (Fonnum *et al.*, 2006) and have been associated with a range of adverse health effects including effects on the nervous system (Seegal *et al.*, 1996) and the

immune system (Nakanishi *et al.*, 1985; Arkoosh *et al.*, 1994), as well as reproductive abnormalities, fetal toxicity, carcinogenicity and interference with the endocrine system (Safe, 1994; Fischer *et al.*, 1998). The production of PCBs was banned in the late 1970s (Dreiem *et al.*, 2009). Until this they were used in the industry as cutting oils, flame retardants, in transformers and capacitors, as well as for many other purposes (Safe, 1994). However, due to their persistence and ability to bioaccumulate and biomagnify through the food chain, humans are still exposed to PCBs mainly through consumption of contaminated food (Dreiem *et al.*, 2009).

PCBs are small molecules that consist of two phenyl rings connected by a carbon-carbon bond, with chlorine substitutions at one or more of the ten available carbons in the molecule (Wright and Welbourne, 2002).

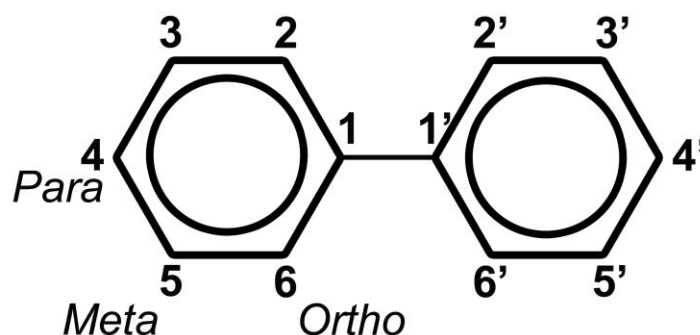


Figure 1-1. General molecular structure of the polychlorinated biphenyls. PCBs may be chlorinated in all positions except 1 and 1', and are named according to the number and position of the chlorine substitutions. Adapted from Wright and Welbourn (2002).

There are 209 possible congeners of PCBs that differ in the number and placement of their chlorine substitutions (Seegal *et al.*, 1996). The PCBs are lipid soluble, and in general their lipid solubility and size of the molecule increases with the degree of chlorination and congener number (Wright and Welbourne, 2002). PCBs were combined in commercial mixtures and marketed according to the chlorine content of the mixture. For example, the brand name Aroclor 1242 meant that the product contained 42 % chlorine by weight (Safe, 1994).

The PCBs are generally divided into three groups according to their structure. The first group comprises the coplanar, dioxin-like PCBs that have chlorine substitution only in the *para* and *meta* positions of the molecule and no substitutions in the *ortho* positions. The second group includes the mono-*ortho*-chlorinated PCBs, which may attain a coplanar configuration, and the third group the PCBs that have two or more *ortho*-substitutions. The latter are always non-coplanar (Mariussen and Fonnum, 2006).

The first two groups have been found to have dioxin-like properties, binding to the aryl hydrocarbon (Ah) receptor and inducing CYP1A1 enzymes, and are associated with carcinogenicity (Safe, 1994). The third group has low affinity for the Ah receptor, but has been associated with effects on the nervous system (Seegal *et al.*, 1996). Also the non-*ortho*-substituted coplanar PCBs have been associated with effects on the nervous system, but they are thought to be less potent in this respect than the *ortho*-substituted (Mariussen and Fonnum, 2006). Exposure to PCBs during development and nursing may predispose to hyperactivity disorders, as well as impairment of learning and memory (Mariussen and Fonnum, 2006). Non-coplanar PCBs have also shown to cause activation of neutrophil granulocytes. This has not been observed for the coplanar PCBs (Fischer *et al.*, 1998; Voie *et al.*, 2000).

Approximately 135 of the 209 PCB congeners have been found in environmental samples (Seegal *et al.*, 1996). PCBs that are present in the environment are slowly biotransformed, and coplanar forms may be converted to non-coplanar forms. Eventually, a higher proportion of non-coplanar congeners is found in the environment than what was originally found in the commercial mixtures. The PCBs detected in human blood and tissues, as well as in wildlife and fish, are mainly non-coplanar congeners (Fischer *et al.*, 1998).

The PCBs used in this thesis are presented in figure 1.2.

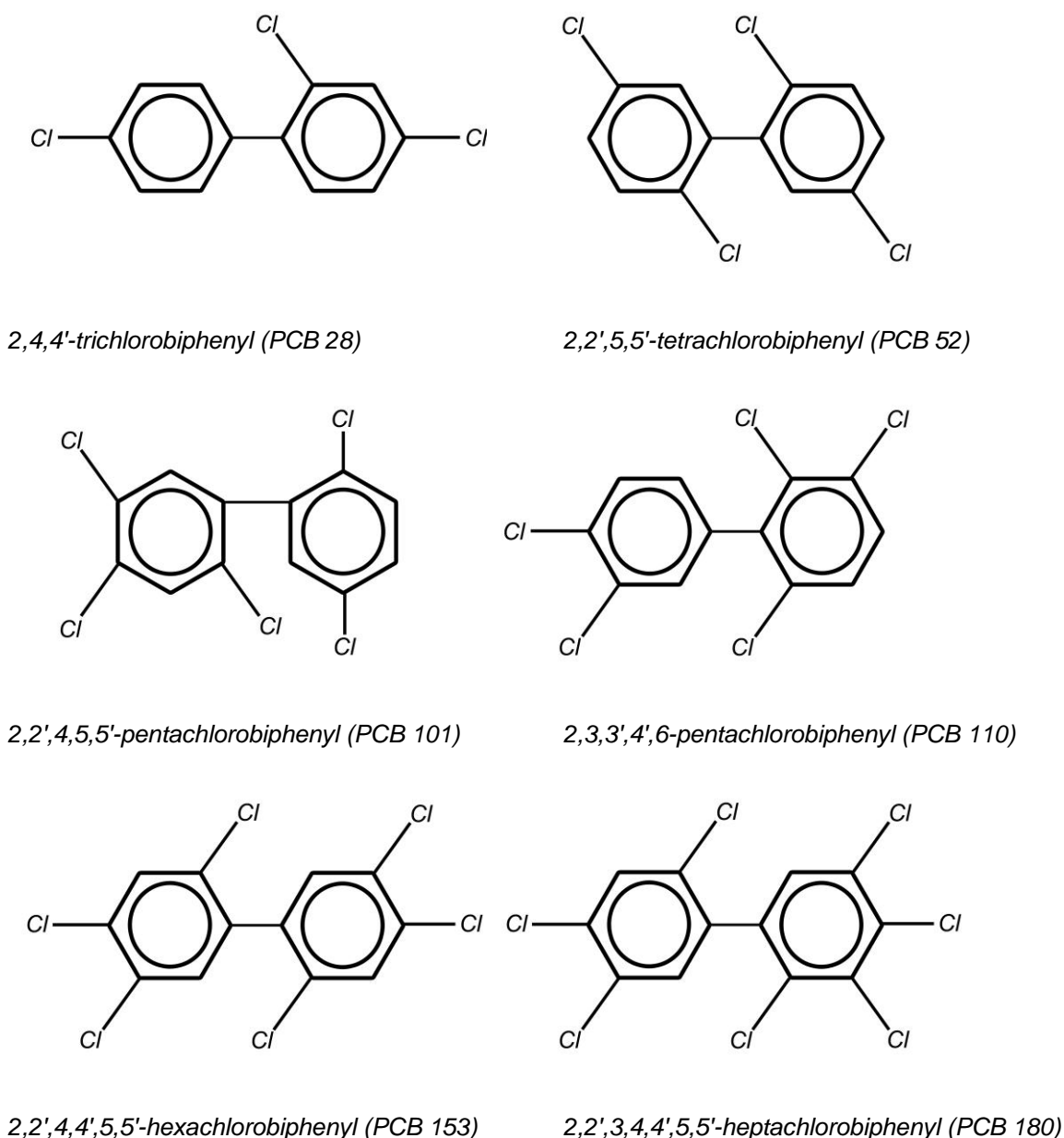


Figure 1-2. Molecular structures of the ortho-chlorinated PCBs 28, 52, 101, 110, 153 and 180.

1.3 Penitrem A

Substances produced by certain moulds growing on food are capable of producing intoxication in man and animals (Newberne, 1974). These so-called mycotoxins are secondary metabolites that are not necessary for the survival of the moulds. They may be produced in situations where they give the organism a competitive advantage. Certain mycotoxins, including penitrem A, are capable of inducing neurological disorders in vertebrates including convulsions, tremor and ataxia (Moldes-Anaya *et al.*, 2009).

The structure and chemical characteristics of the different mycotoxins exhibit a great deal of variation. Most of the tremorogens that have been found to be involved in neuromycotoxicosis contain an indole alkaloid unit in their structure (Moldes-Anaya *et al.*, 2009). Penitrem A is a potent indole diterpenoid (Moldes-Anaya *et al.*, 2010) belonging to a group of mould contaminants known as tremorogenic mycotoxins (Sobotka *et al.*, 1978). It is produced mainly by the fungus *Penicillium crustosum*, and has been associated with neurological symptoms and even death in animals (Moldes-Anaya *et al.*, 2010). Neurological symptoms in man, including tremor, have been reported on a few occasions after the ingestion of *Penicillium crustosum* (Cole *et al.*, 1983; Lewis *et al.*, 2005). Further investigation on the effect of tremorogenic moulds on human health is required due to the probable underreporting of such cases in man (Moldes-Anaya *et al.*, 2010).

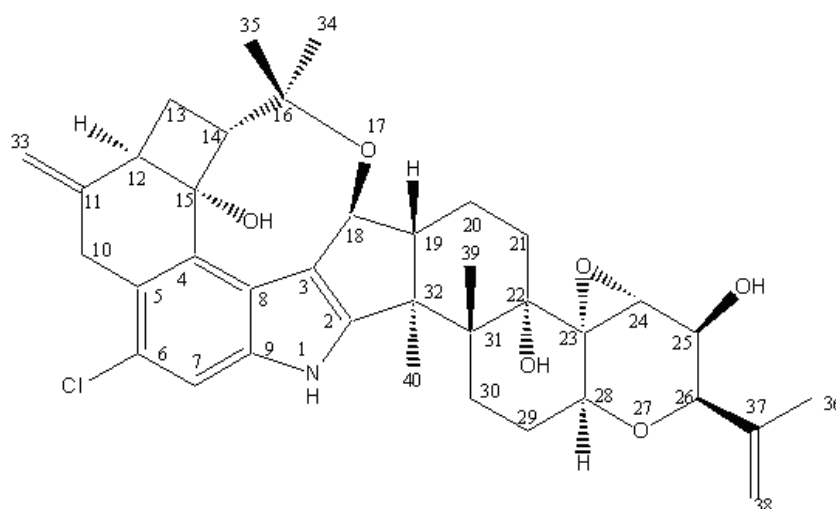


Figure 1-3. Molecular structure of penitrem A. After Moldes-Anaya (2010), with permission.

Penitrem A is able to cross the blood brain barrier (Moldes-Anaya *et al.*, 2009; Eriksen *et al.*, 2010), and signs in intoxicated animals appear within 30 minutes to 3 hours after exposure. The mechanisms responsible for the neurological symptoms are not well understood, but may include activation of the glutamatergic system, interference with the GABAergic inhibitory system as well as blockage of high conductance calcium activated potassium channels (BK channels) in the presynaptic neuronal membrane. (Moldes-Anaya *et al.*, 2010).

1.4 Reactive oxygen species and oxidative stress

1.4.1 Formation of reactive oxygen species

Free radicals have been defined by Halliwell and Gutteridge (2007) as “any species capable of independent existence that contains one or more unpaired electrons”. The fact that they have unpaired electrons makes some of them highly reactive. Many of the molecules defined as reactive oxygen species (ROS) are free radicals, of which some examples are hydroxyl radicals ($\cdot\text{OH}$), superoxide radicals ($\text{O}_2^{\cdot-}$) and nitric oxide (NO^{\cdot}). The term, however, also includes derivatives of oxygen that are not radicals, such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O_3).

Reactive species that contain chlorine, bromine, sulphur and nitrogen in addition to oxygen are included in the term as well, although they could also be described as reactive chlorine, bromine, sulphur and nitrogen species, respectively. Some examples are peroxynitrite (ONOO^-), and the above mentioned NO^{\cdot} and HOCl . The reactivity of the different ROS, and the molecules with which they react, vary between the different species. ROS such as $\text{O}_2^{\cdot-}$, NO^{\cdot} and H_2O_2 react fast and very selectively with certain biological molecules. $\cdot\text{OH}$, however, is not so specific and reacts rapidly with most molecules (Halliwell and Gutteridge, 2007).

ROS are products of the normal cell metabolism in the presence of oxygen. Most are produced through oxidative metabolism in mitochondria, enzymatic mixed-function oxidation reactions and autooxidation of small molecules (Simonian and Coyle, 1996). An overview of ROS production in the cell is illustrated in figure 1.4.

$\text{O}_2^{\cdot-}$ is formed by reduction of molecular oxygen, and may be created inside the cell when high energy electrons leak from the electron transport chain in the mitochondria. It may also be formed by the action of several enzymes located in the cytosol, plasma membrane or nuclear membrane. Such enzymes include phospholipase A_2 (PLA_2), xantine oxidase and NADPH oxidase in phagocytes (Simonian and Coyle, 1996; Halliwell and Gutteridge, 2007). $\text{O}_2^{\cdot-}$ generation is thought to happen within all aerobic cells. This ROS is not thought to be as reactive as e.g. $\cdot\text{OH}$, and does not react with most biological molecules in aqueous fluids (Halliwell and Gutteridge, 2007). $\text{O}_2^{\cdot-}$ may be converted to ONOO^- by reacting with nitric

oxide (NO^\bullet) or to H_2O_2 by the action of superoxide dismutase (SOD) (Simonian and Coyle, 1996).

NO^\bullet is a stable free radical, that has several functions under physiological conditions both in the nervous system and the vascular system (in these cases often simply referred to as NO). It acts as a neurotransmitter, and is involved in memory functions, blood pressure regulation and killing of foreign organisms (Halliwell and Gutteridge, 2007). It has, however, also been suggested as an important cause of nerve cell death during pathological conditions, possibly through the generation of ONOO^- (Simonian and Coyle, 1996).

ONOO^- is a highly reactive molecule, but is more stable than $^\bullet\text{OH}$ and $\text{O}_2^{\bullet-}$, allowing it to diffuse over greater distances. It may react with and produce alterations in lipids, proteins and DNA. ONOO^- may be converted into $^\bullet\text{OH}$, which may also attack the same molecules (Simonian and Coyle, 1996), and NO_2^\bullet . The latter is of intermediate reactivity (Halliwell and Gutteridge, 2007).

$^\bullet\text{OH}$ is the most potent of the ROS, and has a very short half-life. As mentioned above, it may react with most biological molecules, thus initiating free-radical chain reactions (Betteridge, 2000). In addition to production from ONOO^- , $^\bullet\text{OH}$ may be produced from H_2O_2 via the Fenton reaction displayed below. Other intermediate ROS may be generated in the process (Halliwell and Gutteridge, 2007).



H_2O_2 itself is not so reactive, and is continuously produced in most tissues of the body (Halliwell and Gutteridge, 2007). It is produced along the electron transport chain through autooxidation of small molecules. As mentioned, it may also be produced through conversion of $\text{O}_2^{\bullet-}$ catalysed by SOD (Simonian and Coyle, 1996). H_2O_2 is quite water soluble, and may diffuse within and between cells. It is capable of entering cells through membrane water channels (aquaporins), and probably also by other mechanisms. Even though it has a low reactivity, H_2O_2 can be cytotoxic at high concentrations, and may thus have antibacterial properties. It does not damage lipids, DNA or proteins in its own right, but crosses cell membranes where. Intracellularly, it may be converted to OH^\bullet and HOCl (Halliwell and Gutteridge, 2007).

HOCl is produced from H_2O_2 and Cl^- through the action of myeloperoxidase. The myeloperoxidase enzymes are found in phagocytic cells such as the neutrophil granulocyte. They are located in granules in the cytosol that fuse with phagocytic vacuoles, where HOCl is created that may have cytotoxic effects on engulfed bacteria.

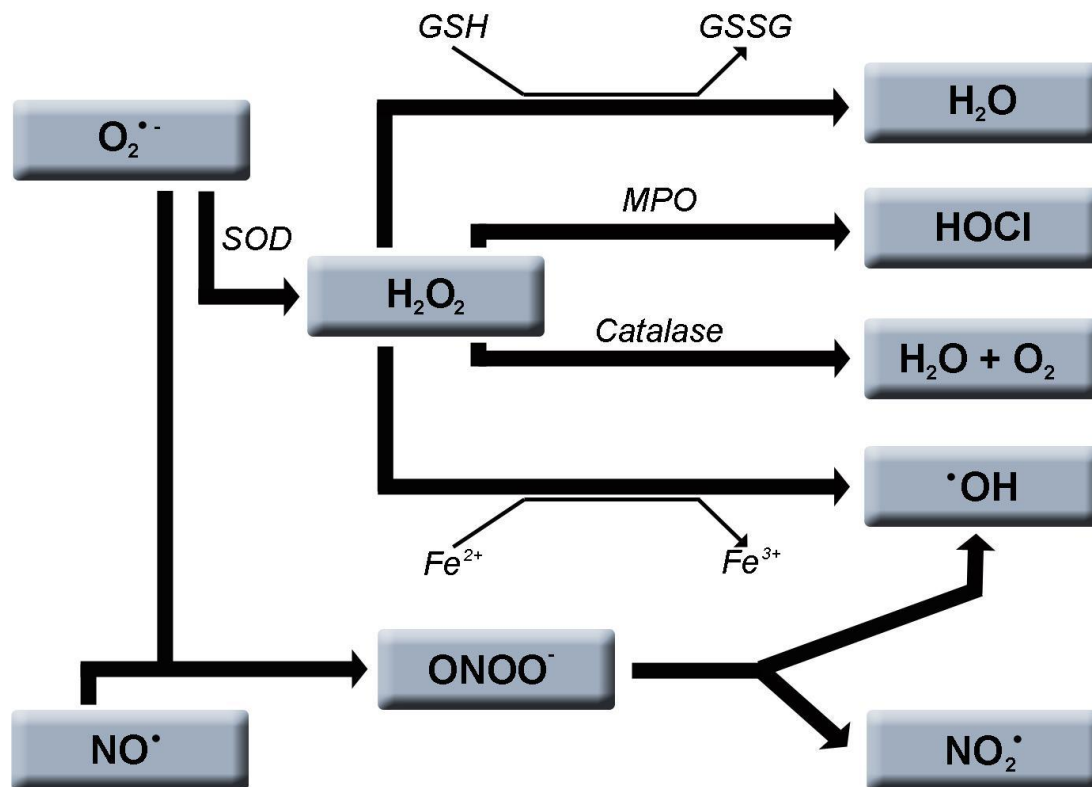


Figure 1-4. An overview of ROS production mechanisms and antioxidant mechanisms in the cell. H_2O_2 may be formed from $\text{O}_2^{\bullet-}$ through the action of superoxide dismutase (SOD). H_2O_2 is converted to water by glutathione peroxidase. GSH and GSSG represent the reduced and oxidised forms of glutathione, respectively. Furthermore, H_2O_2 is converted to HOCl by myeloperoxidase (MPO), and to water and molecular oxygen by catalase. In the presence of iron, H_2O_2 may also be converted to $\cdot\text{OH}$ -radicals through the Fenton reaction. $\text{O}_2^{\bullet-}$ may react with NO^{\bullet} to form peroxynitrite (ONOO^-), which may further be converted to NO_2^- and $\cdot\text{OH}$. Adapted from Rykken (2004).

H_2O_2 and $\text{O}_2^{\bullet-}$ are produced in the body both physiologically and as unavoidable by-products. They may be created where molecules in the body react with oxygen, or, as mentioned above, when electrons escape from the mitochondrial electron transport chain. Physiological ROS formation takes place in phagocytes. H_2O_2 and $\text{O}_2^{\bullet-}$ are involved in phagocyte functions, including killing of invading pathogens such as bacteria and fungi and inactivation of viruses. However, inappropriate activation of phagocytes may also produce deleterious effects, as observed in chronic inflammatory diseases (Halliwell and Gutteridge, 1997).

1.4.2 Antioxidant systems and oxidative stress

The formation of ROS in the body is counterbalanced by antioxidant defence systems. These systems include both intrinsic body functions, and antioxidants supplied through food. Inside the cell, SOD converting $O_2^{\bullet -}$ to H_2O_2 acts in parallel with enzymes that remove H_2O_2 , such as catalase and glutathione peroxidase (GSH-Px), and convert the ROS to water (Halliwell, 1997). Other examples of antioxidant defence systems include iron binding and storage proteins such as transferrin and ferritin, that prevent the creation of damaging $\bullet OH$ radicals by binding iron (Halliwell, 1997). Furthermore, ceruloplasmin binds copper ions and oxidises iron without the creation of ROS, and also prevents lipid peroxidation. Vitamin E and reduced glutathione (GSH) are preferentially oxidised by ROS, thus scavenging ROS that might otherwise cause damage to important biomolecules (Simonian and Coyle, 1996; Halliwell and Gutteridge, 2007).

If the balance between ROS production and the cell defence mechanisms is upset, oxidative stress may occur. This imbalance may result in cellular dysfunction or even cell death, because of damage to molecules such as lipids, proteins and DNA (Simonian and Coyle, 1996). ROS have been suggested as an important factor contributing to cell damage during inflammation, ischemia, cancer and aging (Myhre *et al.*, 2003), and are possibly also involved in neurodegenerative diseases of the brain (Beal, 1995).

Several kinds of stimuli, including environmental contaminants, may induce high levels of ROS formation in the cell, causing them to enter a state of oxidative stress (Finkel and Holbrook, 2000; Myhre *et al.*, 2003). Whether the cell survives or not depends on its ability to withstand stress and repair damaged molecules. If the damage is large enough, the cell will enter apoptosis or necrosis (Simonian and Coyle, 1996).

Increased levels of ROS in the cell may potentially activate specific intracellular pathways (Finkel and Holbrook, 2000). Some of the main stress signalling pathways/mediators activated by oxidant injury are the extracellular signal-regulated kinase (ERK) pathway, the c-Jun amino-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK) signalling cascades, the phosphoinositide 3-kinase (PI(3)K/AKT) pathway, the nuclear factor (NF)- κB signalling system, as well as the p53 and the heat shock response pathways. p53, JNK and p38 activation is most often associated with apoptosis after oxidant injury, whereas

the rest act pro-survival. These pathways are not uniquely involved in oxidative stress, but also in other stress responses, as well as growth and metabolism regulation (Finkel and Holbrook, 2000).

1.4.3 Oxidative stress in the brain

Glutamate is the most prominent excitatory neurotransmitter in the brain. Excitotoxicity refers to the death of neurones due to excessive activation of excitatory amino acid receptors (Beal, 1995; Simonian and Coyle, 1996). This it is an important factor in neuronal degeneration after acute conditions such as hypoxia, ischemia and trauma (Simonian and Coyle, 1996).

Intracellular calcium homeostasis is crucial for the maintenance of normal cell function. Disruption of the homeostasis and increased intracellular levels of free calcium may potentially lead to the production of ROS. Increased Ca^{2+} -levels may also affect neurotransmitter release and activation of phosphokinases, phosphatases and phospholipases. Furthermore, it may affect protease activities, apoptotic processes, and the activity of nitric oxide synthase (NOS) (Mariussen and Fonnum, 2006).

The brain is a region of the body that is particularly vulnerable to oxidative stress. Among the reasons for this are low levels of the enzymes catalase, SOD and GSH-Px. High contents of iron in certain brain areas may also catalyze the formation of $\cdot\text{OH}$. The nerve cell membranes also have a high content of polyunsaturated fatty acids that are particularly vulnerable to ROS-induced damage, such as lipid peroxidation and impairment of membrane function (Mariussen *et al.*, 2002).

Formation of ROS in cerebellar granule neurons has been thought mainly to be the result of release from mitochondria and activation of neuron specific enzymes (Coyoy *et al.*, 2008). Increases in intracellular free calcium may result in the activation of NOS, causing the formation of $\text{NO}\cdot$, as well as the activation of protein kinase C (PKC) and phospholipase A2 (PLA_2), leading to the formation of $\text{O}_2^{\cdot-}$. In addition, calcium may cause the opening of mitochondrial transition pores with subsequent increases in the production of $\text{O}_2^{\cdot-}$ (Fonnum *et al.*, 2006).

Coyoy *et al.* (2008) found that cerebellar granule cells contain an NADPH oxidase system homologous to the one present in phagocytic cells, and that NADPH inhibitors caused decreased $O_2^{\cdot -}$ production. This suggested a role for the NADPH oxidase in ROS production also in cerebellar granule cells. They also postulated a role of PKC in its activation (Coyoy *et al.*, 2008).

1.5 Cerebellar granule cells

1.5.1 Cerebellar granule cells as a model

The cerebellum is a part of the brain that is involved in the control of movements. Granule cells are the most abundant neurons in the cerebellum, and are suited as a model for *in vitro* studies of effect of toxic substances on nerve cells (Gallo *et al.*, 1982). Together with the Purkinje cells they are the most important targets in the cerebellum for toxic substances.

During brain development, different cell types develop at different times. The cerebellar granule cells develop post-natally, and are formed on day 7-15 with a peak at days 10-11, as compared to the Purkinje cells, which are formed *in utero* (Fonnum and Lock, 2000). The fact that the cells develop post-natally makes them relatively easy to isolate, and cultures may be produced from rat or mouse pups from day 7 or 8 after birth. Furthermore, it is possible to obtain a quite pure culture of these neuronal cells by adding cytosine arabinofuranoside (ARA-C), which prevents the replication of glial cells (Gallo *et al.*, 1982).

The cerebellar granule cells are also well suited for *in vitro* studies of cellular and molecular mechanisms for survival and cell death (Reistad *et al.*, 2006). The benefit of using primary cultures of nerve cells instead of cell lines, is the fact that intracellular pathways are kept intact. The cells do not undergo transformations that may potentially alter cell activities, as one may see in cell lines (Aam, 2007).

Cerebellar granule cells have glutamatergic receptors where excitotoxicity may be induced, involving increases in free intracellular calcium and ROS formation (Fonnum and Lock, 2000). As PCBs have been shown to act via the NMDA receptor and to produce increases in

ROS in cerebellar granule cells, these cells are suited for studies on PCB neurotoxicity and PCB-induced ROS formation (Mariussen and Fonnum, 2006).

There have been no published studies on the effects of penitrem A in cerebellar granule cells. However, post mortem examination of dogs that have been intoxicated with penitrem A reveals extensive damage to the cerebellum (Moldes-Anaya, 2010), but not to any other parts of the brain. This selective damage to the cerebellum was also found in *in vivo* experiments by Breton *et al.* (1998) and Cavanagh *et al.* (1998). Breton *et al.* (1998) found an extensive loss of Purkinje cells that was hypothesised to have resulted from excitotoxicity due to the activation of glutamatergic receptors. Cavanagh *et al.* (1998) also found discrete foci of necrosis in the granular cell layer in addition to large losses of Purkinje cells. Due to the selective toxicity of penitrem A to the cerebellum, it was considered that it might yield interesting results to study exposure of cerebellar granule cells to penitrem A.

1.5.2 The MTT assay for detection of cell death

Several different methods are available for the measuring survival in cells during cytotoxicity experiments. Certain methods are based on counting cells after inclusion or exclusion of a dye (e.g. the trypan blue exclusion assay) or measurement of released ^{51}Cr -labeled protein after cell lysis (Mosmann, 1983).

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is a method of measuring the activity of living cells via mitochondrial dehydrogenases (Sigma Aldrich, n.d.). It is based on the cleavage of the yellow tetrazolium salt MTT into the blue coloured product formazan by the mitochondrial enzyme succinate-dehydrogenase. The conversion happens only in living cells, and the amount of formazan produced is proportional to the number of living cells present (Denizot and Lang, 1986). The resulting formazan may be dissolved in an appropriate solvent, and values measured spectrophotometrically. A decrease in the number of living cells will cause a change in the amount of formazan produced, and indicate the degree of cytotoxicity caused by the toxicant (Sigma Aldrich, n.d.).

The method was chosen in preference to the trypan blue assay, which has traditionally been used in PCB toxicity studies (Dreiem *et al.*, 2009), because trypan blue is classified as a possible human carcinogen (International Agency for Research on Cancer, 1998).

Furthermore, the MTT assay is rapid to perform, and is not based on subjective evaluation of cell numbers, as the trypan blue assay is.

1.6 Human neutrophil granulocytes

1.6.1 ROS formation in human neutrophil granulocytes and choice of model

Human neutrophil granulocytes are an important part of the innate immune system protecting us from invading pathogens, as well as an important factor in acute inflammatory reactions. During an immune response, neutrophil granulocytes form ROS aimed at killing microorganisms (Fonnum *et al.*, 2006). When neutrophil granulocytes ingest pathogens via phagocytosis they increase their consumption of molecular oxygen. This is called the respiratory burst. An activated form of the enzyme NADPH oxidase is assembled in the part of the plasma membrane that will form the phagocytic vacuole (Halliwell and Gutteridge, 2007).

$O_2^{\cdot -}$ and H_2O_2 are produced from molecular oxygen through donation of electrons from the NADPH oxidase, and may either be released extracellularly or into the phagocytic vacuole where they may kill certain pathogens (Dahlgren and Karlsson, 1999). Further conversion into HOCl, ONOO⁻ and possibly $\cdot OH$ and O_3 promotes further pathogen killing. MPO may also be released extracellularly where it causes formation of HOCl (Halliwell and Gutteridge, 2007). Although the respiratory burst is important for the defence against pathogens, it may also cause deleterious effects such as tissue destruction during chronic inflammatory reactions (Dahlgren and Karlsson, 1999; Halliwell and Gutteridge, 2007).

Single ortho-substituted PCBs and PCB mixtures such as Aroclor 1242 have shown to induce ROS formation in human neutrophil granulocytes (Voie *et al.*, 1998; Voie *et al.*, 2000; Myhre *et al.*, 2003; Myhre *et al.*, 2009). For this reason, neutrophil granulocytes were chosen for the study of PCBs also in this case. Human neutrophil granulocytes make good models for the study of ROS production after toxicant exposure, as they are both easy to isolate and available in great numbers.

There have been no published studies on effects of penitrem A in human neutrophil granulocytes. However, as it was observed in the cerebellar granule cell experiments that the antioxidant vitamin E gave protection against penitrem A-induced cell death, it was found interesting to see if penitrem A would induce ROS production in human neutrophil granulocytes. Cavanagh *et al.* (1998) described changes resembling ischemic damage in the cerebellum after *in vivo* penitrem A toxicity studies in rats. As mentioned, ROS have been suggested as an important factor contributing to cell damage during ischemia (Myhre *et al.*, 2003).

1.6.2 Detection of reactive oxygen species *in vitro*

For the most complete detection of ROS formation in biological systems such as the human neutrophil granulocytes, a combination of different techniques should be used. There are different techniques available that are specific for different kinds of ROS. Two techniques that may be used are the 2',7'-dichlorodihydrofluorescein (DCF) assay and the 5-amino-2,3-dihydro-1,4-phthalazindione (luminol)-amplified chemiluminescence assay (Myhre *et al.*, 2003).

2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) is a nonpolar, non-ionic probe that diffuses into the cell through the cell membrane, and is hydrolyzed by intracellular esterases to the nonfluorescent compound DCFH. DCFH may be oxidized to its strongly fluorescent form DCF in the presence of ROS (LeBel *et al.*, 1992; Myhre *et al.*, 2003). DCF is often used as a general indicator of oxidative stress in cells, as it detects the presence of various ROS (Wang and Joseph, 1999). Myhre *et al.* (2003) found that it was sensitive to oxidation by ONOO^- , $\cdot\text{OH}$, H_2O_2 in the combination with cellular peroxidases, and peroxidases alone. $\cdot\text{OH}$ and ONOO^- may act within seconds or minutes, whereas other oxidants require more time (Myhre *et al.*, 2003).

Luminol is a dye that is able to penetrate the cell membrane. It releases energy in the form of a blue light when it is being excited by ROS (Dahlgren and Karlsson, 1999). This light may be measured in a luminometer and used as a measure of ROS formation. Chemiluminescence in the presence of luminol is dependent on the myeloperoxidase- H_2O_2 - Cl^- system (Myhre *et*

al., 2003; Halliwell and Gutteridge, 2007) and detects the presence of HOCl (Myhre *et al.*, 2003).

1.6.3 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is a cytoplasmic oxidoreductase, which converts pyruvate to lactate in the presence of reduced nicotinamide adenine dinucleotide (NADH). Leakage of this enzyme is a useful marker for cell damage (Koh and Choi, 1987). The LDH assay has been used to assess integrity of human neutrophil granulocytes (Reistad and Mariussen, 2005; Reistad *et al.*, 2005; Aam and Fonnum, 2007). LDH released from cells may be assessed in an assay where lactate, NAD^+ , resazurin and diaphorase are supplied. Lactate is converted to pyruvate through the action of LDH, and NAD^+ is reduced to NADH. Resazurin is then converted to the fluorescent resorufin by diaphorase, and NADH is oxidized to NAD^+ . Fluorescence may then be measured in a fluorometer. The generation of resorufin is proportional to the amount of extracellular LDH present (Promega Corporation, 2009).

1.7 Cytoprotective agents and inhibitors of ROS production

To elucidate mechanisms involved in cytotoxicity of cerebellar granule cells, as well as ROS formation in human neutrophil granulocytes *in vitro*, different pharmacological substances may be used. In this study, the following substances were used in the cerebellar granule cell PCB 52/180-experiments: (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801), 1-amino-3,5-dimethyladamantane (memantine), cyclosporine A (CsA), 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126), N- ω -nitro-L-arginine methyl ester (L-NAME), α -tocopherol (vitamin E) and 1,2-bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM).

In the cerebellar granule cells studies involving penitrem A, the above mentioned substances were also used, with the exception of memantine. Additional substances tested with penitrem A include 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX), glycine, diazepam, gamma-aminobutyric acid (GABA), bicuculline, phenobarbital, pentobarbital, alphaxalone, allopregnanolone, 4-[4-fluorophenyl]-2-[4-methylsulfinylphenyl]-5-[4-pyridyl]-

1H-imidazole (SB203580), anthra[1,9-cd]pyrazol-6(2H)-one (SP600125) and tacrolimus (FK-506).

In the human neutrophil granulocyte PCB experiments the following substances were used: CsA, U0126, vitamin E, FK-506, SB203580, SP600125 and BAPTA-AM. The same substances, as well as L-NAME, were used for penitrem A granulocyte experiments.

L-glutamate is the main excitatory neurotransmitter in the brain. In addition to metabotropic receptors, there are three kinds of ionotropic glutamate receptors. The latter include the N-methyl-D-aspartate (NMDA) receptor, which contributes a slow component to the excitatory synaptic potential, the α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor, and the kainate receptor involved in fast excitatory synaptic transmission (Rang *et al.*, 2007).

Memantine and MK-801 are antagonists of the NMDA receptor ion channel (Wong *et al.*, 1986; Bormann, 1989). Memantine is less potent than MK-801 (Weller *et al.*, 1993). NBQX is a selective antagonist of the AMPA and kainate receptors (Sheardown *et al.*, 1990). The NMDA receptor requires glycine in addition to glutamate to be activated. Glycine also acts as an inhibitory neurotransmitter, primarily in the spinal cord, and also to some extent in the brain stem, where it acts on glycine receptors (Rang *et al.*, 2007).

GABA is the main inhibitory neurotransmitter in the brain. There is one ionotropic GABA receptor called the GABA_A receptor, which has got several subtypes, and one metabotropic receptor called the GABA_B receptor. The GABA_A receptor has several binding sites for centrally acting drugs (Rang *et al.*, 2007), which are presented in figure 1.5.

GABA itself is the most important endogenous agonist in the body, acting on the GABA binding site (Johnston, 1996). Binding of GABA to its site will open the ion channel within the receptor and allow for influx of Cl⁻ ions, hyperpolarizing the cell (Rang *et al.*, 2007). Bicuculline is a convulsant that blocks synaptic inhibition by GABA receptors by antagonising the action of GABA at the GABA binding site (Johnston, 1996; Rang *et al.*, 2007).

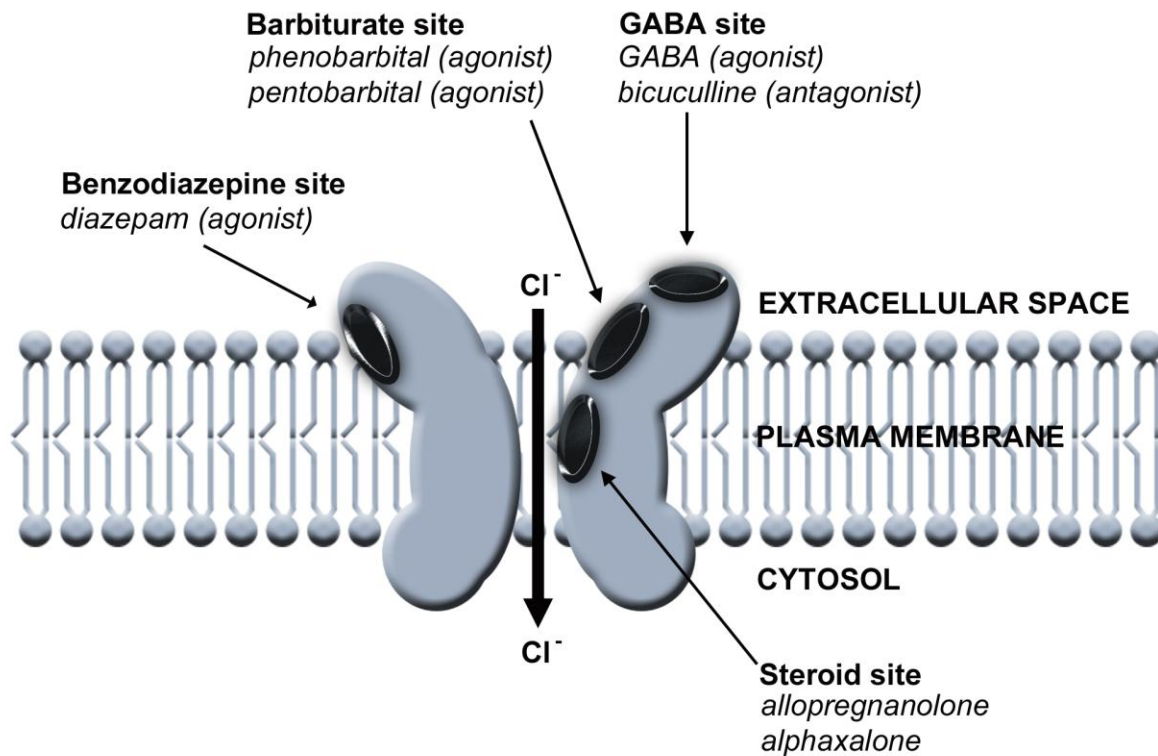


Figure 1-5. The GABA receptor with binding sites for agonists and antagonists used in the cerebellar granule cell experiments. Adapted from Moldes-Anaya (2010).

GABA_A receptors contain several sites where allosteric modulators may bind that affect the effect of the agonist (Johnston, 1996). Benzodiazepines, such as diazepam, potentiate the effect of GABA on the GABA_A receptor. Binding of these agonists to the benzodiazepine site facilitates the binding of GABA to its site (Rang *et al.*, 2007) and increases the opening frequency of the chloride channels within the receptor (Johnston, 1996). Barbiturates such as pentobarbital and phenobarbital increase the mean duration of the opening of the GABA_A receptor chloride channels (Rang *et al.*, 2007). Neurosteroids, such as alphaxalone and allopregnanolone, in general enhance the activation of GABA_A by GABA by increasing the average opening time of the chloride channels, and also by increasing the opening frequency (Johnston, 1996).

The fat-soluble vitamin E is a minor constituent of biological membranes. It is thought to be important in the preservation of membrane integrity by protecting polyunsaturated fatty acids of the membrane from oxidation. Its antioxidant action is fast and non-enzymatic and its prime role is believed to be scavenging lipid peroxyl radicals (Wang and Quinn, 2000). It has also been found to be involved in the scavenging of singlet oxygen ($^1\text{O}_2$) (Kaiser *et al.*, 1990).

and reactions with ONOO^- (Hogg *et al.*, 1994) and $\text{O}_2^{\cdot-}$ (Ha and Csallany, 1992). Vitamin E has also been found to inhibit PKC (Wang and Quinn, 2000).

L-NAME is a nitric oxide synthase (NOS) inhibitor (Moncada and Higgs, 1991).

Five MAPK families have been identified in mammals. These are ERK 1/2, ERK 3/4, JNK, p38 and ERK 5 (Schaeffer and Weber, 1999; Chen *et al.*, 2005). U0126 is a specific inhibitor of the MEK1/2 that phosphorylate and activate ERK1 and ERK2 (Favata *et al.*, 1998). It also inhibits MEK 5, which acts upstream of ERK 5 (Kamakura *et al.*, 1999). SB203580 is a cell permeable, selective inhibitor of the p38 pathway (Cuenda *et al.*, 1995), whereas SP600125 is a cell permeable, potent, reversible inhibitor of JNK (Bennett *et al.*, 2001). FK-506 and CsA are immunosuppressant agents that complex with an immunophilin and inhibit the protein phosphatase calcineurin (Schreiber and Crabtree, 1992; Matsuda *et al.*, 1998). They also inhibit the p38 and JNK pathways (Matsuda *et al.*, 2000). CsA also inhibits opening of mitochondrial transition pores in the inner mitochondrial membrane (Bernardi *et al.*, 1994).

BAPTA-AM is an ester of the Ca^{2+} chelator BAPTA. Due to the presence of four acetomethyl (AM) groups in the molecule, BAPTA-AM may cross the cell membrane. Once in the cytosol, esterases cleave the AM groups, and the chelator is effectively trapped inside the cell where it may bind intracellular free Ca^{2+} and inactivate PKC (Dieter *et al.*, 1993; Ndountse and Chan, 2009).

1.8 Aim of study

The aim of this study was to assess whether there was a difference in neurotoxicity in cerebellar granule cells between low and highly chlorinated *ortho*-substituted PCBs *in vitro*, and to identify possible factors involved in their neurotoxicity. The neurotoxic potential of penitrem A was also investigated along with potential factors involved. Further it was investigated if there was a difference in the ability of the PCBs to induce ROS production in human neutrophil granulocytes *in vitro*, and to identify possible pathways involved in this induction. The ROS production after penitrem A exposure was also assessed, as was potential pathways involved.

2. Materials and methods

2.1 Chemicals

2.1.1 Chemicals for the cultivation of cerebellar granule cells and the MTT assay:

Chemical	Abbreviation	Producer
Albumin from bovine serum, lyophilized powder ≥ 96 % (agarose gel electrophoresis)	BSA	Sigma-Aldrich, St. Louis, MO, USA
Basal medium Eagle (1X), liquid, with Earle's salt, without L-glutamine	BME	Invitrogen (GIBCO), Norway
Cytosine β -D-arabinofuranoside – crystalline	ARA-C	Sigma-Aldrich, St. Louis, MO, USA
Deoxyribonuclease I from bovine pancreas	DNase	Sigma-Aldrich, St. Louis, MO, USA
Dimethyl sulfoxide, bioreagent for molecularbiology, $\geq 99.9\%$	DMSO	Sigma-Aldrich, St. Louis, MO, USA
Foetal bovine serum (heat inactivated)	FBS	Invitrogen, (GIBCO) Norway
Glutamax-I supplement, 200 mM		Invitrogen, Norway
Penicillin-streptomycin, liquid, 100 IU/ml penicillin, 100 μ g/ml streptomycin		Invitrogen, Norway
Phenol red, powder, bioreagent, cell culture tested		Sigma-Aldrich, St. Louis, MO, USA
Poly-L-lysine hydrobromide, molecular weight $>70,000$		Sigma-Aldrich, St. Louis, MO, USA
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide	MTT	Sigma-Aldrich, St. Louis, MO, USA
Trypsin type I from bovine pancreas, 10,000 BAEE units/mg protein		Sigma-Aldrich, St. Louis, MO, USA

Trypsin inhibitor from Glycine max (soybean), type I-S lyophilized powder		Sigma-Aldrich, St. Louis, MO, USA
---	--	-----------------------------------

2.1.2 Chemicals for the isolation and experiments with human neutrophil granulocytes:

Chemical	Abbreviation	Producer
Dextran		
2',7'- dichlorodihydrofluorescein diacetat	DCFH-DA	Invitrogen, Norway
Hanks' Balanced Salt Solution (10X), liquid	HBSS	Invitrogen (GIBCO), Norway
HEPES buffer solution 1M, liquid	HEPES	Invitrogen, (GIBCO) Norway
5 amino-2,3-dihydro-1,4-phthalazindione $\geq 97\%$ (HPLC)	Luminol	Sigma-Aldrich, St. Louis, MO, USA
Lymphoprep, density gradient medium		Medinor, Norway
Methanol, Chromasolv, gradient grade, for HPLC, $\geq 99.9\%$		Sigma-Aldrich, St. Louis, MO, USA
CytoTox-ONE		Promega, Madison, WI, USA
TRITON-X		Promega, Madison, WI, USA

2.1.3 Test chemicals

Chemical	Systematic (IUPAC) name	Abbreviation	Producer
Synthetic vitamin E $\geq 96\%$ (HPLC)	α -tocopherol	Vitamin E	Sigma-Aldrich, St. Louis, MO, USA
BAPTA-AM (intracellular Ca^{2+} chelator) $\geq 95\%$ (HPLC)	1,2-bis(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester	BAPTA-AM	Calcbiochem, San Diego, USA

Cyclosporine A - ≥98.5% (TLC)	(3S,6S,9S,12R,15S,18S,21S,24S,30S)-30-ethyl-33-[(1R,2R,4E)-1-hydroxy-2-methylhex-4-en-1-yl]-1,4,7,10,12,15,19,25,28-nonamethyl-6,9,18,24-tetrakis(2-methylpropyl)-3,21-bis(propan-2-yl)-1,4,7,10,13,16,19,22,25,28,31-undecaazacyclotritriacontan-2,5,8,11,14,17,20,23,26,29,32-undecone	CsA	Sigma-Aldrich, St. Louis, MO, USA
Tacrolimus	5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26aS-hexahydro-5S,19R-dihydroxy-3S-[(1E)-2-(4R-hydroxy-3R-methoxycyclohexyl)-1R-methylethenyl]-14S,16S-dimethoxy-4R,10,12S,18R-tetramethyl-8R-(2-propenyl)-15R,>99%	FK-506	CAYMAN, Michigan, USA
Glycine	Glycine		Sigma-Aldrich, St. Louis, MO, USA
Memantine hydrochloride	1-amino-3,5-dimethyladamantane ≥ 98% (GC)		Sigma-Aldrich, St. Louis, MO, USA
MK-801 (BK channel blocker)	(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate	MK-801	Sigma-Aldrich, St. Louis, MO, USA
SB203580 (p38 MAP kinase pathway inhibitor)	4-[4-fluorophenyl]-2-[4-methylsulfinylphenyl]-5-[4-pyridyl]-1H-imidazole	SB203580	Calcbiochem, San Diego, USA
SP600125 (JNK inhibitor II)	Anthra[1,9-cd]pyrazol-6(2H)-one	SP600125	Calcbiochem, San Diego, USA

U0126	1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene	U0126	Promega, Madison, WI, USA
L-NAME	N- ω -nitro-L-arginine methyl ester	L-NAME	Sigma-Aldrich, St. Louis, MO, USA
NBQX disodium salt	2,3-dihydroxo-6-nitro-7-sulfamoyl-benzo(F)quinoxaline	NBQX	Tocris Biosciences, Bristol, UK
Penitrem A \geq 95% (TLC)			Sigma-Aldrich, St. Louis, MO, USA
Diazepam	7-chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2(1H)-one		Sigma-Aldrich, St. Louis, MO, USA
GABA	γ -aminobutyric acid		Sigma-Aldrich, St. Louis, MO, USA
Bicuculline methochloride \geq 99 %	[R-(R*,S*)]-5-(6,8-dihydro-8-oxofuro[3,4-e]-1,3-benzodioxol-6-yl)-5,6,7,8-tetrahydro-6,6-dimethyl-1,3-dioxolo[4,5-g]isoquinolinium chloride		Tocris Biosciences, Bristol, UK
Phenobarbital	5-ethyl-5-phenyl-2,4,6-pyrimidinetrione, 5-ethyl-5-phenylbarbituric acid		Sigma-Aldrich, St. Louis, MO, USA
Pentobarbital sodium salt	5-ethyl-5-(1-methylbutyl)-2,4,6-trioxohexahydropyrimidine		Sigma-Aldrich, St. Louis, MO, USA
Alphaxalone \geq 99 %	(3 α ,5 α)-3-hydroxypregnane-11,20-dione		Tocris Biosciences, Bristol, UK

Allopregnanolone	(3 α ,5 β)-3-hydroxy-pregnan-20-one		Tocris Biosciences, Bristol, UK
Ethanol			Sigma-Aldrich, St. Louis, MO, USA

The ortho-substituted PCBs 28, 52, 101, 110, 153 and 180 were gifts from Patrick Andersson, Department of Chemistry, University of Umeå, Sweden. The PCBs had been chemically purified to remove any traces of coplanar, dioxin-like PCBs such as PCB 126.

All other reagents used were analysis grade laboratory chemicals from standard commercial suppliers.

Stock solutions of the PCBs and penitrem A, were prepared by dissolution in DMSO. Allopregnanolone, alphaxalone, CsA, FK-506, U0126, SB203580, SP600125, BAPTA-AM and vitamin E were also dissolved in DMSO. L-NAME was dissolved in PBS with pH 7.4. NBQX, pentobarbital and bicuculline were dissolved in distilled water, whereas MK-801, memantine and glycine were dissolved in Millique water. Phenobarbital was dissolved in Ethanol.

2.2 Laboratory animals

Rats used for cultivation of cerebellar granule cells were at the start of the study purchased from Scanbur, Sweden. However, after a viral infection in the rat population of the supplier, animals were for the rest of the study purchased from Tactonic, Denmark.

Mixed sex litters of ten Wistar rat pups, 7 days of age, were delivered with a surrogate mother at the day of cell culture preparation. The rats were housed in the animal department for no longer than 2 hours prior to euthanasia.

The surrogate mother was euthanized by the use of CO₂ and decapitation. The rat pups were decapitated without the prior use of anaesthesia.

2.3 Cerebellar granule cell experiments

2.3.1 Cultivation of cerebellar granule cells

Primary cultures of cerebellar granule cells were prepared largely corresponding to the method described by Gallo *et al.* (1982).

Each week, on the day preceding cell cultivation, appropriate solutions (1-4) were prepared (see appendix), and sterilely filtrated through 0.22 µm filters (Sarsted, Nümbrecht, Germany). 12-well multi-dish plates (Nunc) were pre-treated for at least 1 hour with 1 ml per well of 0,01mg/ml poly-L-lysine solution to ensure attachment of the cells to the bottom of the wells. 500 ml of medium for cell cultivation was prepared, and kept in the refrigerator for up to 4 weeks. This consisted of basal Eagle's medium supplemented with 10 % heat inactivated foetal bovine serum, 2,5 mM Glutamax, 100 IU/ml penicillin + 100 µg/ml streptomycin (P/S), 25 mM potassium chloride (KCl) and 1% glucose. All the above stages were carried out aseptically in a laminar air flow (LAF) cabinet (Holten Laminar Air).

At the day of cell cultivation, the 7 day old rat pups were euthanized at the animal department premises by decapitation. The heads were kept on ice, and dipped in 70 % ethanol prior to removal of the cerebellum. When dissected out the cerebellum was put in a sterile 50 ml conical red top tube (Sarsted, Nümbrecht, Germany) with 10 ml solution 1, containing magnesium sulphate (MgSO₄) and bovine serum albumin (BSA).

Back at the laboratory, the rest of the cultivation procedure was carried out aseptically in the LAF cabinet. Meninges and blood vessels were dissected away from the cerebellum, and the clean tissue put in fresh solution 1. The cerebelli were cut in 3-4 pieces using a scalpel blade and transferred to a 15 ml conical red top tube (Sarsted, Nümbrecht, Germany). The tube was then centrifuged for 1 minute at 1000 revolutions per minute (rpm) in a table centrifuge (Heraeus, Megafuge 1.0).

After centrifugation the supernatant was removed, and 10 ml solution 2 containing trypsin was added. The re-suspended cerebellar pieces and solution were then transferred to a 250 ml Erlenmeyer flask, which was placed in an incubator shaker (Innova 4000) for 15 minutes at approximately 60 rpm and 37 °C. This trypsinization stage was carried out to dissolve adhesions between cells in order to obtain individual cells for the next stages of cultivation.

It was important that the trypsinization was not extended for longer than indicated, as this could have caused damage to the cells.

After the end of the trypsinization, the dissolved cerebellar tissue was removed from the flask with as little solution 2 as possible. It was then re-suspended in a new 15 ml tube, in approximately 10 ml of solution 4 (a dilution of solution 3), containing trypsin inhibitor and deoxyribonuclease (DNase). The trypsin inhibitor ends the action of trypsin, whereas the DNase prevents lumping of DNA released from cells dying during the cultivation process. The tube was then centrifuged for 1 minute at 1000 rpm. This stage was repeated if necessary, until there was a clear distinction between the pellet and the supernatant.

In the following step the pellet was re-suspended in 3 ml of solution 3, and homogenization of the pellet was carried out by the use of a series of glass Pasteur pipettes, on which the openings had been made progressively smaller by the use of a bunsen burner. When a homogenous solution had been achieved, the tube was filled with 10 ml pre-prepared cell culture medium and centrifuged for 7 minutes at 900 rpm.

The resulting pellet was then dissolved in 2 ml of medium and transferred to a 150 ml flask containing further medium. 125 ml medium was used for 10 rat pups and 10 multi-well dishes, ensuring 1 ml cell suspension per well, and a concentration of approximately $1-1,2 \times 10^6$ cells per ml. A 5 ml Pasteur pipette was used to transfer cells to the plates. The flask was rotated and the content mixed thoroughly every time a new volume was withdrawn. After being transferred to the plates, the cells were placed in an incubator at 36 °C, in a 5 % CO₂ atmosphere, for 7-9 days before being used in experiments.

18-24 hours after placement in the incubator, 25 µl cytosine arabinofuranoside (ARA-C) dissolved in basal medium Eagle (BME) was added to each well, giving a concentration in the medium of 10,3 µM. ARA-C prevents the proliferation of non-neuronal glial cells, and ensures a relatively pure culture of granule cells.

2.3.2 *In vitro* toxin exposure of cerebellar granule cells

After 7 days of incubation the cerebellar granule cells were used for *in vitro* cell survival experiments with PCB and penitrem A. Preliminary studies were carried out with varying concentrations of PCB 52 and PCB 180 at 6, 12, 18 and 24 hours (results are not displayed)

before it was decided that cell survival experiments were to be carried out with 24 hours exposure to the toxins. For assessment of a dose-response relationship, exposure to the following doses was included: 2.5, 5, 10, 12.5, 15, 20 and 30 μ M PCB 52 and 180.

In experiments aimed at elucidating mechanisms involved in PCB 52 and PCB 180 cytotoxicity, incubation of PCBs was carried out with potentially neuroprotective substances. The following substances and concentrations were tested: 3 μ M MK-801, 3 μ M memantine, 0.5 μ M CsA, 50 μ M vitamin E, 10 μ M U0126, 300 μ M L-NAME, 1 μ M BAPTA-AM, and 5 μ M BAPTA-AM. The concentrations of the potentially neuroprotective substances were selected based on what was found in the literature.

Each of these substances was tested in conjunction with 20 μ M PCB 52 and PCB 180. Due to the fact that 20 μ M PCB 180 alone caused quite high toxicity, it was chosen to test the neuroprotective agents considered most likely to give effect also in conjunction with 15 μ M PCB 180. This was done because it was considered likely that the high degree of cell death caused by 20 μ M PCB 180 could be difficult to influence by adding protective agents.

In addition to the above mentioned studies of PCB 52 and PCB 180, cells were exposed to 10, 12.5 and 15 μ M PCB 28, 52, 101, 110, 153 and 180 for 24 hours. This was done to assess differences in survival between cells exposed to the same concentration of different PCBs, and difference in cell survival exposure to different concentration of the same PCB.

Cerebellar granule cells were also incubated for 24 hours with 2, 10, 12.5, 15, 20 and 40 μ M penitrem A, in order to assess if there was a dose-response relationship. Also for penitrem A, incubation with neuroprotective substances was carried out for 24 hours to try to identify potential mechanisms involved in its toxicity. Each substance was tested in conjunction with 12.5 μ M penitrem A. As there have been no published studies of penitrem A toxicity in cerebellar granule cells, the concentrations of the substances tested were largely based on the concentrations of the same protective agents used in studies of PCB in cerebellar granule cells. The following substances and concentrations were used: 5 and 50 μ M diazepam, 10 and 50 μ M GABA, 150 and 300 μ M pentobarbital, 300 and 600 μ M phenobarbital, 2 μ M allopregnanolone, 2 μ M alphaxalone and 50 μ M bicuculline, 50 μ M glycine, 20 μ M NBQX, 3 μ M MK-801, 0.5 μ M CsA, 10 μ M U0126, 50 μ M vitamin E, 300 μ M L-NAME, 1 μ M SB203580, 10 μ M SP600125, 1.5 μ M FK-506, 0.5, 5 and 10 μ M BAPTA-AM.

Cerebellar granule cells were also exposed to 12.5 μ M penitrem A for 2, 6, 8, 12 and 24 hours, in order to see if there was a time-related increase in toxicity. A DMSO control, 12.5 μ M penitrem A and 12.5 μ M penitrem A with 50 μ M vitamin E were tested in each experiment.

In each experiment, the different drug concentrations or combinations of drug and neuroprotective agent were tested in triplicate and an average value was calculated for each triplicate. At least two triplicates of controls only containing medium and two triplicates of controls containing dimethyl-sulphoxide (DMSO) were always included in the experiments. As PCBs and certain protective agents were dissolved in DMSO, DMSO controls were included to ensure that the solvent did not exert any toxic effects on the cerebellar granule cells in its own right during an experiment. The amount of DMSO included in the DMSO control wells corresponded to the highest concentration cells were exposed to due to addition of PCBs and/or protective agents. The DMSO concentration never exceeded 0.3%, as a higher concentration could be toxic to the cells (Mariussen E., personal communication 2009).

Little variability was found in cell survival between control wells both within and between plates when assessed by the MTT assay (described in 2.5). Therefore, results were analysed together for all 120 wells (10 plates), and each well was treated as a separate instance regardless of which one of the 12 plates it was located on.

15 ml red top conical tubes, containing 3.5 ml medium, were prepared prior to the experiment and heated in an incubator shaker. The medium was prepared as described in section 2.3.1, but without foetal bovine serum. Serum was not included as albumin may will bind PCB and reduce the response. (Fonnum F., personal communication, 2009) The appropriate amounts of the test substances were added to a tube containing 3.5 ml medium in order to obtain the desired concentration in the control wells. The tube was then vortexed vigorously just prior to application to prevent test substances from sticking to the sides of the plastic tube. When inhibitors were included, these were added at the same time as the toxins.

During the experiment, plates were removed one at the time from the 36°C, 5% CO₂ incubator, and exposed to the toxins and inhibitors dissolved in medium. The original medium from the wells was removed carefully from 3 wells at the time, and 1 ml test solution applied immediately to each well to prevent the wells from drying out. Once this

had been carried out for the whole plate, it was put back into the incubator for the determined time period. The procedure was carried out aseptically in the LAF cabinet.

2.3.3 MTT cell survival assay

Once the cells had been exposed to the toxins and neuroprotective substances for the appropriate amount of time, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used for measurement of cell survival. The assay was carried out according to the protocol developed by Tim Mosmann (1983), with modifications.

0,5 mg/ml of yellow MTT solution with 1 % glucose was prepared by dissolution of the solid powder in phosphate buffered saline (PBS), and sterilely filtrated through 0,22 µm filters (Sarsted, Nümbrecht, Germany) in the LAF cabinet. Appropriate amounts of solution were transferred into sterile 50 ml conical red top tubes, wrapped with aluminium foil and frozen.

At the day of the assay, tubes of MTT solution were thawed in the incubator shaker and pre-warmed to 37° C before addition to the cell culture plates. As during the exposure stage described in 2.3.2, one plate at the time was removed from the 36° C, 5 % CO₂ incubator. Medium was removed carefully from 3 wells at the time using a glass Pasteur pipette and a Pipetboy, and 0.5 ml MTT solution added to the wells. All plates were then returned to the incubator for exactly one hour before they were transferred to a fume hood. The MTT solution was then removed and 0.5 ml DMSO added to the wells. A few minutes were allowed for the purple formazan salts that had been created to be completely dissolved in DMSO. 100 µl of the resulting purple coloured solution were pipetted in duplicate from each well onto a 96-well transparent, flat bottom tissue culture plate (Sarstedt, USA).

Absorbance values were then obtained spectrophotometrically by the use of a multi-well ELISA reader (LabSystem Multiscan Biochromatic, type 348). Dual wavelength measurements were performed at 570 nm (test wavelength) and 690 nm (reference wavelength). Values from the reference wavelength were subtracted from the test wavelength by the machine to obtain the final absorbance values. The plates were read as soon as possible after completion of the experiment.

2.4 Human neutrophil granulocyte experiments

2.4.1 Isolation of human neutrophil granulocytes

Isolation of human neutrophil granulocytes for reactive oxygen species (ROS) measurements was carried out by dextran sedimentation and density gradient centrifugation, as described by Bøyum *et al.* (1991). Blood from different healthy, non-smoking, male volunteers was collected in the morning on the day of the experiment. Approximately 50 ml of blood were withdrawn from each subject. EDTA coated Vacuette tubes (Med-Kjemi AS, Norway) were used to prevent coagulation of the blood.

The blood was divided into two 50 ml conical red top tubes, and 1 ml 6% dextran in 0.9% NaCl per 10 ml blood was added to each tube. The tubes were turned gently a few times and allowed to stand for 30 minutes at room temperature for the erythrocytes to settle at the bottom of the tubes. After sedimentation of the erythrocytes, approximately 10 ml of leukocyte rich supernatant was removed from the tubes and transferred to two 15 ml conical red top tubes. Subsequently, 3 ml of the isoosmotic separation medium Lymphoprep was added to the bottom of each tube using a long 21 gauge 150 mm needle. The tubes were centrifuged at 1900 rpm for 15 minutes at room temperature in a table centrifuge (Heraeus, Megafuge 1.0). This caused the formation of a layer of monocytes at the interface between the supernatant and the separation fluid, whereas a pellet consisting of neutrophils and remaining erythrocytes was formed at the bottom of the tube.

In the next step supernatant, separation medium and the layer of monocytes, was carefully removed by the use of a plastic pipette. The cells were re-suspended in 5 ml 0.83 % NH_4Cl , and left at room temperature for 7 minutes for lysis of the remaining erythrocytes. The tubes were then centrifuged for 7 minutes at 1900 rpm. If the lysis of red blood cells at this stage was incomplete, the preceding step was repeated as many times as considered necessary.

After centrifugation the supernatant was removed, and each of the two pellets was re-suspended in 5 ml 0.9% NaCl washing solution. The tubes were centrifuged again at 1900 rpm for 7 minutes, the supernatant removed, and the two pellets dissolved in 6 ml 1x Hanks' Balanced Salt Solution (HBSS) buffer supplemented with 4.17 mM NaHCO_3 , 20 mM HEPES and 5 mM glucose (from now on only referred to as HBSS buffer). The resulting 12 ml of cell suspension were then transferred to a single 15 ml conical red top tube, and the

number of cells counted in a coulter counter (Coulter Electronics Ltd., Harpenden, Herts, England). The cells were then diluted in HBSS buffer to a concentration of 2.5 million cells/ml buffer. The diluted cells were kept at room temperature, or in the refrigerator if time was long from isolation of cells to the start of the experiments.

2.4.2 Measurement of reactive oxygen species after exposure to toxic substances

ROS formation may be measured by several techniques, of which two are the DCF and the luminol amplified chemiluminescence assays. After preparation as described in 2.4.1, cells were exposed to different concentrations of the 3 ortho-substituted PCBs (PCB 52, PCB 153 or PCB 180), and to the mycotoxin penitrem A. In addition 20 μ M of the 3 different PCBs and 10 μ M penitrem A were tested in combination with different chemicals known to inhibit intracellular pathways leading to the formation of ROS. These inhibitors were also tested alone to elucidate effects on basal ROS production in unstimulated cells.

Stock solutions of the toxic substances PCB and penitrem A, as well as the potential inhibitors of ROS production, CsA, FK-506, U0126, SB203580, SP600125, BAPTA-AM and vitamin E were prepared by dissolution in DMSO. L-NAME was dissolved in PBS with pH 7.4. Different concentrations of toxic substances, combinations of toxic substances and ROS inhibitors as well as ROS inhibitors alone were added to white 96 microwell plates (Nunc A/S Denmark) in triplicate prior to the addition of cells.

The final concentration of DMSO in each well, after addition of cells and buffer, would be 0.2 or 0.4 % per well in the wells with toxic substance and with toxic substance and inhibitor, respectively. As DMSO seemed to decrease basal ROS levels in un-stimulated control cells substantially, especially in the fluorescence experiments, DMSO controls with the same amount of DMSO as found in the experimental wells (0.5 μ l or 1 μ l) were included in each experiment in triplicate at two different sites of the plate. This was done to be able to correct for the suppressive effect of DMSO on ROS production at the different concentrations of toxic substances/combinations of toxic substances and inhibitors, and for the subsequent calculation of relative fluorescence/chemiluminescence values, as percentage of DMSO control. Much higher levels of fluorescence/luminescence were detected in unstimulated controls than in blank wells. This was assumed to reflect basal ROS production

in the unstimulated neutrophil granulocytes. As values were calculated as percentage of the DMSO controls, the use of the term basal ROS levels will in the following parts of this thesis refer to the level of ROS in cells only treated with DMSO.

In each experiment, three wells only containing HBSS buffer (blank) were included to correct for background fluorescence/luminescence, thus allowing subtraction of the blank value from the results. No positive control was included in the PCB experiments, due to the fact that PCB 153 has been studied before and has been shown to induce ROS production (Reistad and Mariussen, 2005).

In the case of penitrem A, preliminary studies with 10 μ M, were conducted in the same studies as the PCBs. Due to the fact that penitrem A caused significantly higher ROS production than PCB 153 when measured by the DCF assay, no positive control was included in following studies where penitrem A was studied alone. Only preliminary luminol chemiluminescence studies were carried out for penitrem A, as luminescence in cells after 10 μ M exposure was not found to be significantly different from control.

2.4.3 Detection of ROS with the DCF assay

The assessment of ROS by the DCF assay was carried out mainly as described by Myhre *et al.* (2000) and Aam and Fonnum (2006), with slight modifications. 150 μ l of HBSS buffer was added to each well of the 96 micro well plate, except for in wells containing the blank, where 250 μ l buffer was added. The appropriate amount of toxic substance and ROS inhibitors was then added to each well, and the plate covered by aluminium foil. 12 ml of cell suspension was divided into three 15 ml red top tubes, with 4 ml suspension in each tube. Subsequently, 0.16 μ l 5 mM stock solution of DCFH-DA in methanol was added to each tube (0.4 μ l per 1 ml of cells), giving a final concentration of 2 μ M DCFH-DA in the cell suspension. The tubes were then covered with aluminium foil to protect the probe from light, and incubated for 15 minutes at 37°C in a water bath. Subsequently, the tubes were centrifuged for 7 minutes at 1900 rpm, the supernatant removed, and replaced by 4 ml HBSS buffer. The cells were resuspended in the buffer, and 100 μ l of cells were added with a multipipette to all the wells of the plate, with the exception of the wells containing the blank. DCF fluorescence was then measured every other minute for 120 minutes, in a microplate spectrofluorometer (Molecular Devices, SpectraMax Gemini EM), and the area under the

curve (AUC) calculated. Values were for statistical purposes expressed as fluorescence relative to DMSO control, set to 100 %.

2.4.4 Detection of ROS with the luminol chemiluminescence assay

Detection of ROS formation by the luminol-amplified chemiluminescence assay was performed as described by Voie *et al.* (1998) and Aam and Fonnum (2006) with modifications. 50 µl of HBSS buffer was added to each well of the 96 microwell plate, with the exception of the blank triplicate where 250 µl was added. The appropriate amounts of toxic substances and toxic substance/inhibitor combinations were added, and the plate covered with aluminium foil. 10 ml of 0.25 mM luminol in HBSS was prepared in a 15 ml red top tube by dissolution of 25 µl of the 0.1 M stock solution of luminol in DMSO in 9.975 ml HBSS. As luminol is light sensitive, the tube was covered with aluminium foil. 100 µl of luminol solution followed by 100 µl of cell suspension was then added to the microwell plate, with the exception of the blank. The final concentration of luminol in each well was 0.1 mM. Luminescence was measured every other minute for 1 hour in a multidetection microplate reader (BioTek Instrument's Synergy 4 Multidetection Microplate Reader), and the AUC was calculated. Values were for statistical purposes expressed as luminescence relative to DMSO control, which was set to 100 %.

2.4.5 LDH assay

Assessment of cell integrity after exposure of human neutrophil granulocytes to 10 µM penitrem A and 20 µM of the different PCBs was done with the LDH homogenous membrane integrity assay CytoTox-ONE kit from Promega. Leakage of LDH is a useful marker for cell damage (Koh and Choi, 1987). 250 µl of cell suspension consisting of 100 µl cells and 150 µl HBSS buffer, was added to 1.5 ml Eppendorf tubes in conjunction with the toxic substances. Cells were exposed in triplicate for 2 hours as previously described for the DCF assay. Once the incubation time had expired, 200 µl of the cell mixture was transferred to new 1.5 ml Eppendorf tubes, and centrifuged at 15 000 g for in a bench centrifuge at room temperature for 3 minutes. (Sorvall, Refrigerated MicroCentrifuge-14). Subsequently, 100 µl of supernatant was transferred to a white 96 microwell plate, and 100 µl of the kit reagent containing lactate, NAD⁺, reazurin and diaphorase was added in darkness. After 10 minutes

of incubation the reaction was stopped by adding 50 μ l of the kit stop solution, preventing further generation of fluorescent products. Resorufin fluorescence was measured at room temperature, in a multilabel counter (Victor 2, Perkin Elmer) at excitation and emission wavelengths of 530 nm and 580 nm, respectively. Included in the assay were wells only containing buffer (blank), negative control only containing buffers and cells, positive control containing 0.3 % Triton-X-100, and cells incubated with the toxic substances. Triton-X-100, lyses the cell membrane allowing for release of LDH. Values were calculated as % LDH release as compared to control only containing buffer and cells.

2.5 Statistical analyses

In this section choice of statistical methods will be discussed in general. The methods applied in each individual case will be mentioned in the relevant context in the results section. Statistical analyses and calculations were carried out by the use of GraphPad Prism 4, PASW Statistics 18 software and Microsoft Excel 2003. Graphical plots were made in GraphPad Prism 4, and in one case in Microsoft Excel 2003.

Generally, data were analyzed in GraphPad Prism 4, where assessments for deviations from normality and homogeneity of variances between groups were made prior to statistical comparisons. These assessments, as well as the assumption that observations are independent are prerequisites that must be fulfilled prior to the use of ANOVA and its corresponding post hoc tests (Motulsky, 2003).

The assumption that the experimental data come from a population with an approximate normal distribution was tested by use of Dallal and Wilkinson's method (adapted from Lilliefors' modified Kolmogorov-Smirnov test), and graphical depiction in box-plots. The same method was used to assess if the differences between each set of pairs come from a normally distributed population in case of the paired t-test. Dallal and Wilkinson's method does not require prior knowledge of the mean and standard deviation of the overall population, as opposed to the original Kolmogorov-Smirnov test, and is the test for normality applied by GraphPad Prism 4 (Motulsky, 2003). No significant deviation from normality was found in the experiments conducted.

Homogeneity of variances between groups that were to be compared statistically, was in most cases assessed by Bartlett's test. In the few cases where there were less than 4 individual experimental values in one or several of the groups that were to be compared, homogeneity of variances was assessed by use of Levene's test – followed by other appropriate tests in PASW – as GraphPad Prism does not perform the Bartlett's test under such circumstances.

For multiple pairwise comparisons between groups, in the cases where the above mentioned prerequisites were fulfilled, an ANOVA test was conducted and followed by an appropriate post hoc test if the null hypothesis (that there was no significant difference between the groups) was rejected.

For comparison between the mean of a control group and the means of each of the different experimental groups that were tested in the ANOVA, a Dunnet's post hoc test was chosen. Where multiple comparisons of the means of all the groups with each other were of interest a Tukey-Kramer test was performed.

Also in certain cases a post hoc test for the detection of a linear trend was performed. This was used to determine whether the mean values of groups increase or decrease in a systematic manner along the x-axis. The test is based on a linear regression on group mean versus group number (in order of appearance from left to right) on the x-axis (Motulsky, 2003).

In the cases where there was found to be unequal variances between the different experimental groups, multiple pairwise comparisons were conducted by the use of a Welch test. A Games-Howell post hoc test was used if the null hypothesis (that there was no significant difference between the groups) was rejected. This was done in PASW Statistics 18. These tests do not require that variances in the groups are homogenous, and should avoid the increased risk of type 1 errors that may occur if tests that require equal variances are applied to data that violate these assumptions. Such a violation will especially produce a higher chance of type I errors if you have a small number of experiments (Games *et al.*, 1983; Zimmerman, 2004).

As sample sizes are relatively small for the presented data, a non-parametric test such as the Kruskal-Wallis test was not considered to be the best choice when intra-group variances

were unequal. These tests have little power to detect differences when sample sizes are small (Motulsky, 2003).

In all the cases where a toxin was studied with and without the addition of a neuroprotective substance or ROS inhibitor, a two-tailed, paired, Student's t-test was applied to assess statistical differences. As above mentioned, the pairwise differences between treatments were tested for normality before the t-test was conducted. Due to the fact that a large number of neuroprotective substances/inhibitors were tested, it was not always possible to include all in the same experiment. This resulted in some variation in the value for the toxin tested alone, and is the reason why the value for the toxin alone has been presented together with the value for the toxin with each neuroprotective substance/inhibitor when graphically displayed in the results section.

The neuroprotective substances and ROS inhibitors were always also tested alone (without PCBs or penitrem A) to see if they themselves had any significant effect on survival or basal ROS production, respectively. In the case of the cerebellar granule cells, results were compared to control, whereas they were compared to DMSO control in the human granulocyte experiments. This was either done by ANOVA followed by a Dunnet's post hoc test or with a Welch test followed by a Games-Howell test, depending on normality and equality of variances.

In the case of PCB 52 and PCB 180, best fit dose-response curves were obtained from the cerebellar granule cells experiments by non-linear regression in GraphPad Prism 4. The values that would kill half of the cell population (LC_{50} values) were computed for the two PCBs, and the difference between them was tested for statistical significance by the program. For completion of this step concentration values on the x-axis were log transformed, whereas y-values were normalized to a common scale. The dose response curve for penitrem A and its LC_{50} value were obtained in the same manner.

In all the experiments conducted a p-value of < 0.05 was regarded as statistically significant. In the following results section, exact p-values will be reported where this is available, otherwise p-values will be reported as $p < 0.05$, $p < 0.01$ and $p < 0.001$. As a general rule, only values for results that were statistically significant have been reported in the results section, but in certain cases non significant values have been included for completeness.

Group means are reported as well as the standard error of the mean (SE). Confidence intervals are reported where this is found relevant. Where the unit of the mean value is percent of control, SE is given in percentage points, not as a relative percentage of the given mean. For simplicity of reading percentage points are in these cases represented by the symbol “%”. This applies if not otherwise specified.

In the case of LC₅₀ values, deviations from mean are represented by confidence intervals (CI). As the LC₅₀ values are calculated from log values after fitting of the dose response curves it is not appropriate to back-calculate the SE, and GraphPAD Prism therefore reports the mean value and the 95 % confidence interval (Motulsky, 2003).

3. Results

3.1 Neurotoxicity studies in cerebellar granule cells

3.1.1 General considerations

Cerebellar granule cells were exposed in triplicate to toxins and/or potentially neuroprotective substances. Neuroprotective substances were also tested alone (without toxins). Survival was assessed with the MTT assay, and calculated as percentage of control (set to 100 %), which only contained medium. This was done for all the cerebellar granule cells experiments for both PCB and penitrem A.

For the PCB toxicity experiments, none of the neuroprotective substances caused a significant change in survival compared to control when cells were exposed to these alone. This was assessed using an ANOVA with a Dunnet's post hoc test. One of the neuroprotective substances used in conjunction with penitrem A showed significant influence on survival also when used alone. This is discussed in the section on penitrem A and neuroprotective substances (3.1.6).

DMSO controls, corresponding to the highest concentration of DMSO used in the experiment, were included in all experiments and did not cause any significant reduction in survival compared to control. This was the case in all the cerebellar granule cells experiments conducted for both PCB and penitrem A

3.1.2 Comparison of PCB 52 and PCB 180

Cerebellar granule cells were exposed in triplicate to 2.5, 5, 10, 12.5, 15, 20 and 30 μ M PCB 52 and PCB 180 for 24 hours ($n = 6-12$). Both PCB 52 and 180 showed a dose dependent decrease in survival with increasing concentration. For PCB 52 a concentration of 15 μ M and higher showed a statistically significant reduction in survival compared to control, whereas for PCB 180 this was the case for concentrations from 10 μ M and above. Dose response curves for PCB 52 and PCB 180 are presented in figure 3.1.

After exposure to PCB 52, survival was reduced to 46 ± 7 % (mean \pm SE, $p < 0.01$) for 15 μ M, 21 ± 3 % ($p < 0.001$) for 20 μ M and 3.0 ± 0.6 % ($p < 0.001$) for 30 μ M.

For PCB 180, survival was reduced to 45 ± 4 % (mean \pm SE) for 10 μ M, 31 ± 4 % for 12.5 μ M, 17 ± 4 % for 15 μ M, 8 ± 1 % for 20 μ M, and 2.4 ± 0.2 % for 30 μ M ($p < 0.001$ for all). A Welch test with a Games-Howell post hoc test was used to assess statistical differences.

The LC_{50} values for PCB 52 and PCB 180 were estimated to 14.3 (95 % CI [13.7, 15.0]) and 9.5 (95 % CI [8.9, 10.1]) μ M respectively, and found to be significantly different from each other ($p < 0.0001$).

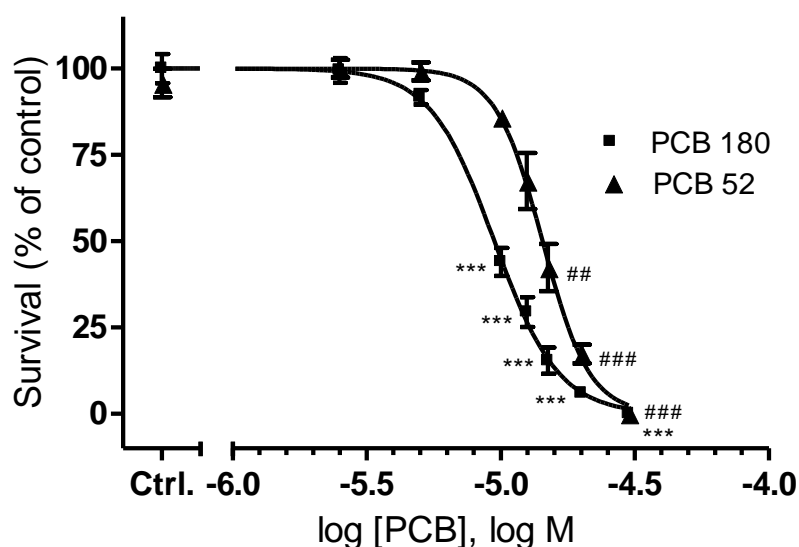


Figure 3-1. Survival of cerebellar granule cells *in vitro* as percentage of control after 24 hours of exposure to 2.5, 5, 10, 12.5, 15, 20 and 30 μ M PCB 52 and PCB 180. The concentration values are log-transformed. Survival data was assessed with the MTT assay. Survival values are presented as mean \pm SE ($n = 6-12$ in triplicate). A Welch test with Games-Howell post hoc test was performed to assess if survival was statistically different from control. For PCB 52, the significance levels are indicated as # = $p < 0.05$, ## = $p < 0.01$ and ### = $p < 0.001$. For PCB 180: * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. LC_{50} values for PCB 52 and 180 were estimated to 14.3 (95 % CI [13.7, 15.0]) and 9.5 (95 % CI [8.9, 10.1]) μ M, respectively.

3.1.3 Effects of potentially neuroprotective substances on PCB 52 and PCB 180 toxicity

Cerebellar granule cells were incubated in triplicate with 20 μ M PCB alone as well as in conjunction with one of several potentially neuroprotective substances for 24 hours. The data were compared with a two-tailed, paired Student's t-test.

20 μ M PCB 52: Average survival after exposure to PCB 52 alone and in conjunction with the different neuroprotective agents is presented in figure 3-2.

For 0.5 μ M CsA, 10 μ M U0126, 300 μ M L-NAME and 3 μ M memantine there was no significant difference between cells exposed to 20 μ M PCB 52 in conjunction with the inhibitor and to PCB alone ($n = 3-5$).

20 μ M PCB with 50 μ M vitamin E ($n = 7$), 3 μ M MK-801 ($n = 5$) and 5 μ M BAPTA-AM ($n = 3$) were significantly different from 20 μ M PCB alone. Vitamin E caused an average increase in survival of 43 (95% CI [30, 57], $p = 0.0002$), MK-801 an increase of 17 (95% CI [9, 24], $p = 0.0032$), and BAPTA-AM a decrease of 24 (95% CI [6, 41], $p = 0.0279$) percentage points.

As mentioned in 3.1.1, neuroprotective substances were also tested on cells alone ($n = 3-5$), but none caused a significant change in survival compared to control.

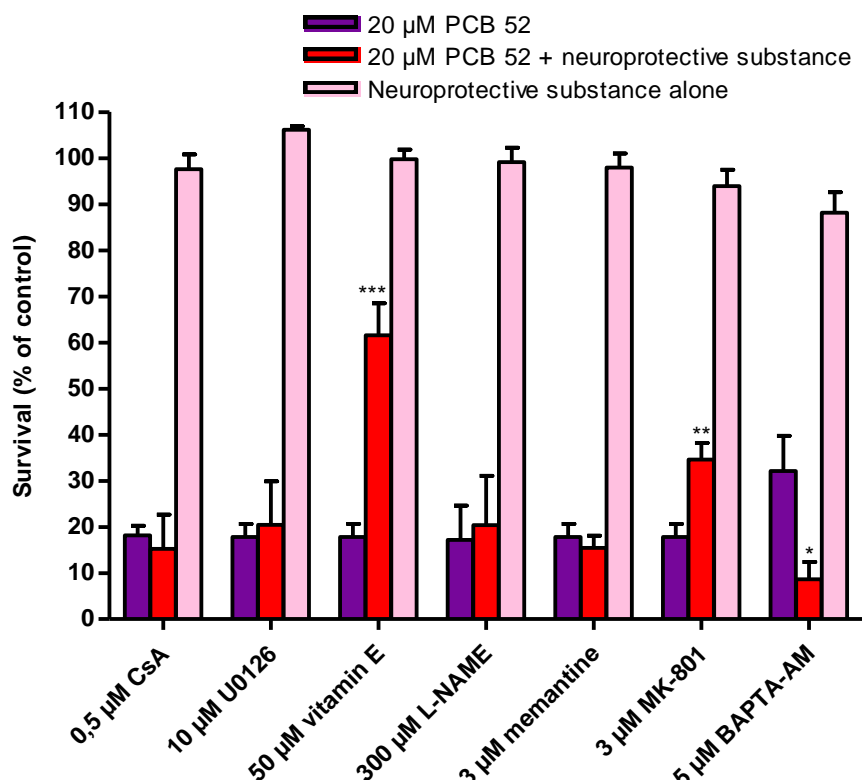


Figure 3-2. Survival of cerebellar granule cells after 24 hours of exposure to 20 µM PCB 52 and potentially neuroprotective substances *in vitro*. Survival was measured with the MTT survival assay. Mean survival of cells and SE are presented as percentage of control ($n = 3-7$ in triplicate). Differences in survival between groups were analysed with a two-tailed, paired Student's *t*-test. The symbols *, **, and *** represent $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Cells were also exposed to neuroprotective substances alone ($n = 3-5$ in triplicate). No significant differences in survival were observed compared to control. An ANOVA with Dunnett's post hoc test was used.

20 µM PCB 180: The average survival in cerebellar granule cells after the exposure to 20 µM PCB 180 with and without neuroprotective agents is shown in figure 3-3.

Survival after incubation of cells with 20 µM PCB 180 alone was not significantly different from survival after incubation in conjunction with 10 µM U0126, 300 µM L-NAME, 3 µM memantine, 3 µM MK-801 or 1 µM BAPTA-AM ($n = 3-7$).

Incubation with 0.5 µM CsA caused a small but statistically significant average increase in survival of 2.2 (95 % CI [0.5, 3.8], $p = 0.0198$) percentage points compared to PCB alone ($n = 6$). Vitamin E with 20 µM PCB 180 caused a significant average increase in survival of 24 (95 % CI [19, 30], $p = 0.0003$) percentage points ($n = 5$).

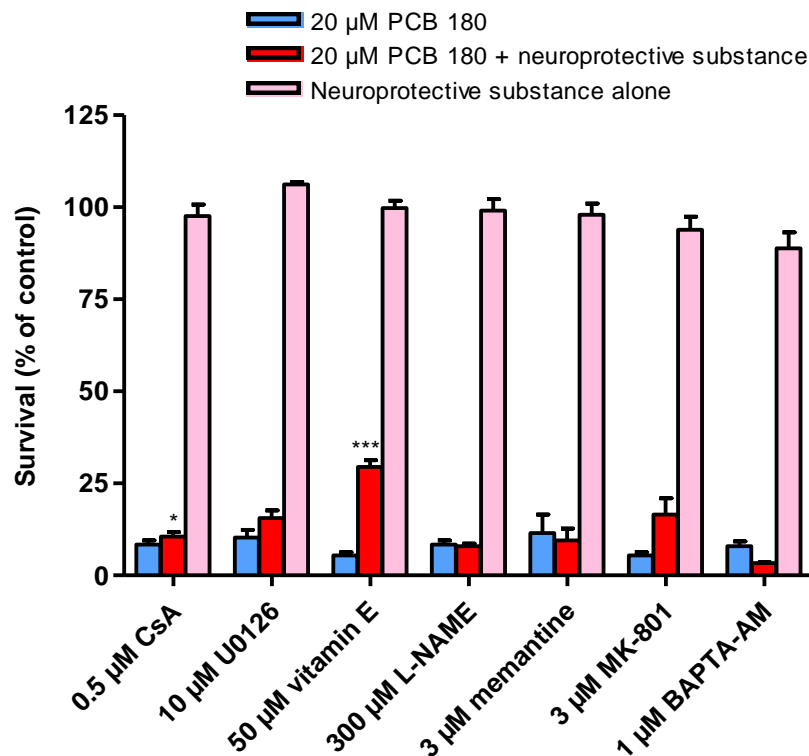


Figure 3-3. Survival of cerebellar granule cells after 24 hours of exposure to 20 µM PCB 180 and potentially neuroprotective substances in vitro. Survival values were measured with the MTT survival assay. Mean survival of cells and SE is presented as percentage of control ($n = 3-7$ in triplicate). Differences in survival between groups were analysed with a two-tailed, paired Student's *t*-test. The symbols *, **, and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. Exposure to neuroprotective substances alone did not cause significant changes in survival compared to control ($n = 3-5$ in triplicate). An ANOVA with Dunnet's post hoc test was used.

15 µM PCB 180: The average survival values for 15 µM PCB 180 with and without inhibitors are displayed in figure 3-4.

There was no statistically significant difference in survival when cells were incubated with PCB and 3 µM memantine or with 15 µM PCB 180 alone ($n = 3$). 5 µM BAPTA-AM ($n = 3$) caused an average decrease in survival of 27 (95 % CI [-1, 55], $p = 0.054$) percentage points, but this was not significant.

PCB 180 in conjunction with 10 µM U0126, 50 µM vitamin E and 3 µM MK-801 all caused significant increases in survival compared to 15 µM PCB 180 alone ($n = 6$). U0126 caused an average increase in survival of 8 (95% CI [1, 14], $p = 0.025$), vitamin E an increase of 28 (95 % CI [18, 37], $p = 0.0007$) and MK-801 an increase of 19 (95 % CI [5, 33], $p = 0.012$) percentage points.

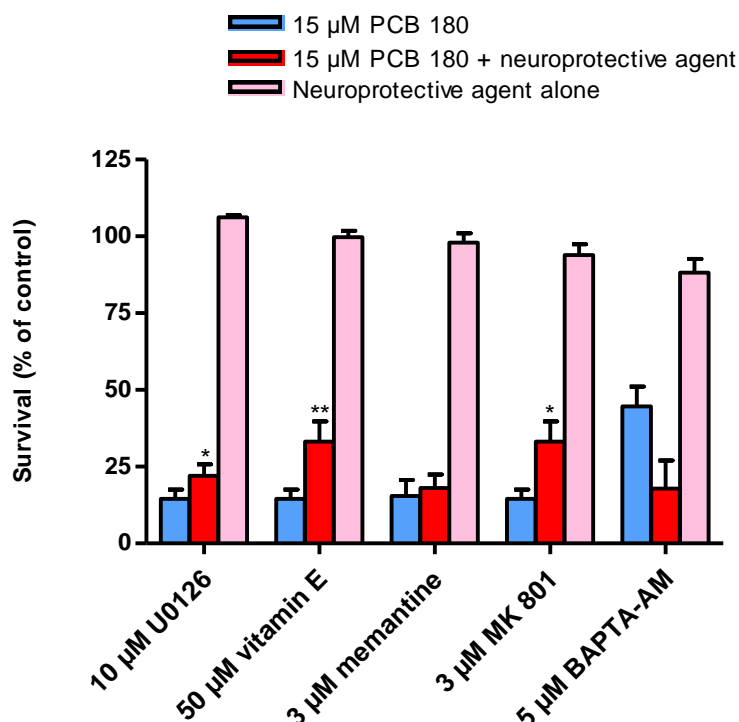


Figure 3-4. Survival of cerebellar granule cells after 24 hours of exposure to 15 µM PCB 180 and potentially neuroprotective substances *in vitro*. Survival values were measured with the MTT survival assay. Mean survival of cells and SE are presented as percentage of control ($n = 3-6$ in triplicate). Differences in survival between groups were analysed with a two-tailed, paired Student's *t*-test. The symbols *, **, and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. Exposure to neuroprotective substances alone did not cause any significant change in survival compared to control ($n = 3-5$ in triplicate). An ANOVA with Dunnet's post hoc test was used.

3.1.4 Neurotoxicity of low and highly chlorinated PCBs

Cerebellar granule cells were exposed to 10, 12.5 and 15 µM solutions of PCBs with increasing congener number ($n = 6-7$). PCB 28, 52, 101, 110, 153 and 180 were tested. For PCBs 52, 110, 153 and 180 there was a dose dependent decrease in survival with increasing concentration. There was no significant decrease in survival for PCB 28 or PCB 101. An ANOVA with Tukey-Kramer post hoc test was used.

None of the concentrations of PCB 28 showed a statistically significant difference in cell survival compared to control (Welch test, $p = 0.061$). Survival in cells exposed to PCB 52 was only significantly different from control for concentrations of 12.5 and 15 µM ($p < 0.05$ and $p < 0.01$, respectively).

After exposure to 12.5 and 15 μM PCB 101, cerebellar granule cell survival was statistically significant from control ($p < 0.01$ for both). For PCBs 110, 153 and 180, concentrations of 10, 12.5 and 15 μM all caused a statistically significant decrease in survival compared to control ($p < 0.01$ for all). Comparison between different concentrations of each PCB and control was done using individual ANOVA and Dunnet's post hoc tests. Results are displayed in figure 3-5.

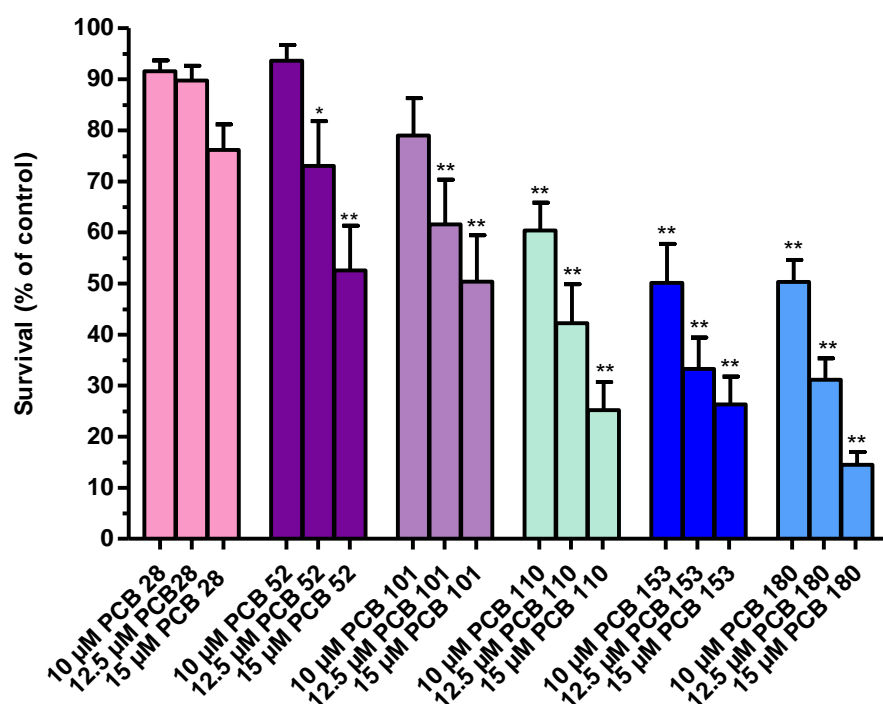


Figure 3-5. Survival of cerebellar granule cells in vitro after 24 hours of exposure to 10, 12.5 and 15 μM of PCB 28, 52, 101, 110, 153 and 180. Survival was measured using the MTT assay and calculated as percentage of control. Survival values are presented as mean + SE ($n = 6-7$ in triplicate). The different concentrations of PCBs were compared to control with a Welch test for PCB 28 and an ANOVA and Dunnet's post hoc tests for PCB 52-180. Significant differences from control are indicated as * = $p < 0.05$ and ** = $p < 0.01$.

In order to determine whether there was a systematic change in mean survival with increasing congener number, the experimental data for each concentration of all PCBs were also analysed with an ANOVA with a post hoc test for a linear trend. There was a significant negative linear trend from PCB 28 to PCB 180 for all concentrations ($p < 0.0001$ for all, $r^2 = 0.60$ for 10 μM , $r^2 = 0.64$ for 12.5 μM and $r^2 = 0.59$ for 15 μM). This indicates that the higher congeners induced more cell death than the lower congeners. To assess differences in survival between equal concentrations of different PCBs, a Tukey-Kramer post hoc test was used. These differences and the linear trend are illustrated in figure 3-6.

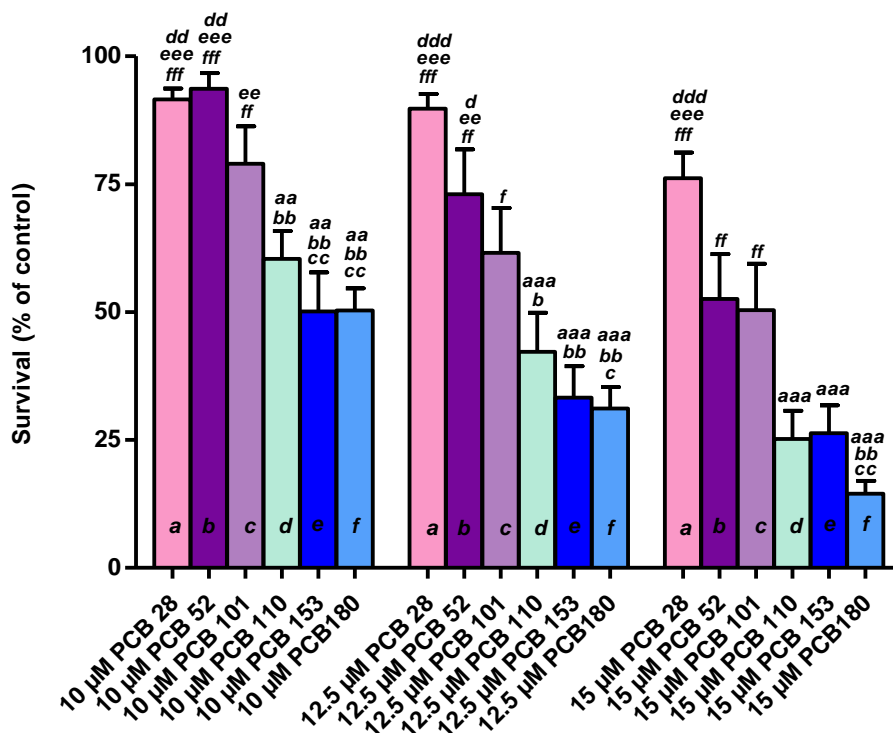


Figure 3-6. Survival of cerebellar granule cells in vitro after 24 hours exposure to 10, 12.5 and 15 µM of PCB 28, 52, 101, 110, 153 and 180, analysed using the MTT assay. Survival is calculated as percentage of control, and presented as mean + SE (n = 6-7 in triplicate). The diagram illustrates the significant negative linear trends that were found for survival with increasing congener number at all concentrations. Comparisons of different PCBs at the same concentrations were performed with an ANOVA and a Tukey-Kramer post hoc test. The letters a, b, c, d, e and f are used to represent PCB 28, 52, 101, 110, 153 and 180, respectively. The letters above the columns indicate that survival for the congener represented by the column was statistically different from survival for the congeners indicated by the letters, when tested at the same concentration. One, two and three letters represent significance levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. I.e., "aa" above 10 µM PCB 180 means that survival in the cells exposed to 10 µM PCB 180 was significantly different from survival in cells exposed to 10 µM PCB 28, with a p -value < 0.01 .

3.1.5 Neurotoxicity of penitrem A at different concentrations

Cerebellar granule cells were exposed in triplicate to 2, 10, 12.5, 15, 20 and 40 µM penitrem A for 24 hours (n = 4-7). There was a dose dependent decrease in survival with increasing concentrations of penitrem A. Survival was found to be significantly different from control at all concentrations, except for 2 µM, and was reduced to 58 ± 5 % (mean \pm SE) ($p < 0.01$) for 10 µM, 22 ± 5 % ($p < 0.001$) for 12.5 µM, 13 ± 4 % ($p < 0.001$) for 15 µM, 6 ± 2 % ($p < 0.001$) for 20 µM, and 1 ± 1 % ($p < 0.001$) for 40 µM.

0.001) for 20 μM and $1.8 \pm 0.2\%$ ($p < 0.001$) for 40 μM . Statistical analyses were done using a Welch test with a Games-Howell post hoc test.

A best fit dose-response curve for penitrem A is presented in figure 3-7. The LC_{50} value estimated for penitrem A from this curve was 10.5 (95 % CI [9.9, 11.1]) μM .

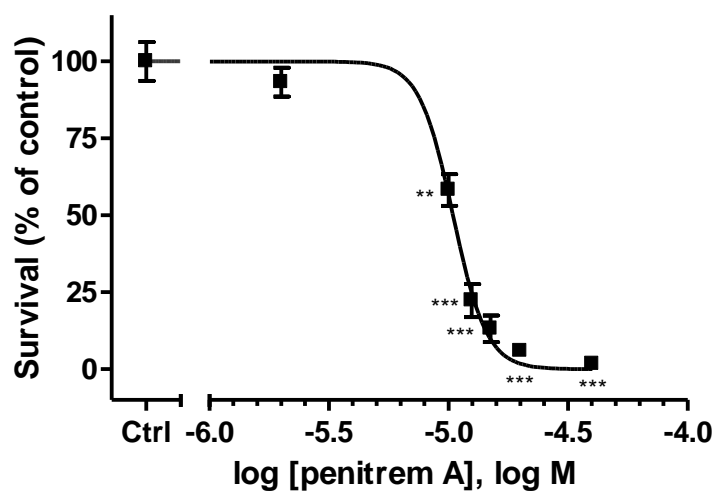


Figure 3-7. Survival of cerebellar granule cells after 24 hours of exposure to 2, 10, 12.5, 15, 20 and 40 μM penitrem A in vitro. Survival was measured with the MTT assay. The concentration values are log-transformed. Values are presented as mean \pm SE in percent of control ($n = 4-7$ in triplicate). A Welch test with Games-Howell post hoc test was performed to detect statistical differences between the different concentrations and control. The symbols *, ** and *** represent $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. LC_{50} for penitrem A was estimated to be 10.5 (95% CI [9.9, 11.1]) μM .

3.1.6 Penitrem A tested with potentially neuroprotective substances

Cerebellar granule cells were exposed to several potentially neuroprotective substances in conjunction with 12.5 μM of penitrem A for 24 hours. For graphic display, the substances have been divided into substances that act primarily extracellularly on receptors (figure 3-8), and substances acting primarily intracellularly (figure 3-9). A two-tailed, paired Student's t-test was used to assess differences in survival between cells exposed to penitrem A alone and in conjunction with neuroprotective substances.

Cells were also incubated with inhibitors alone ($n = 3-6$, except 0.5 μM BAPTA-AM and 50 μM bicuculline [$n = 2$], 2 μM alphaxalone and 2 μM allopregnanolone, [$n = 1$]). No statistics

could be conducted for alphaxalone and allopregnanolone, but the values are included in the graphs for completeness.

When compared to incubation with penitrem A alone, no statistically significant increase in survival was seen for cerebellar granule cells after incubation of 12.5 μ M penitrem A with 5 μ M diazepam, 150 and 300 μ M pentobarbital, 300 μ M phenobarbital, 2 μ M allopregnanolone, 2 μ M alphaxalone, 300 μ M bicuculline, 50 μ M glycine, 3 μ M MK-801, 20 μ M NBQX, 10 μ M U0126, 1 μ M SB203580 or 0.5 μ M BAPTA-AM (n = 3-7).

GABA: Compared to penitrem A alone, there was a small but significant average increase in survival after exposure to 10 μ M and 50 μ M GABA in conjunction with 12.5 μ M penitrem A (n = 6). For 10 μ M GABA the increase was 7 (95 % CI [1,13], $p < 0.024$) and for 50 μ M GABA 2.4 (95 % CI [0.5, 4.2], $p = 0.0198$) percentage points.

Diazepam: 50 μ M diazepam caused a statistically significant increase in survival of 14 (95 % CI [7, 21], $p < 0.01$) percentage points when compared to penitrem A alone (n = 8).

Phenobarbital: 600 μ M phenobarbital caused a statistically significant increase in survival 17 (95 % CI [3, 31], $p = 0.0306$) percentage points compared to penitrem A alone (n = 5).

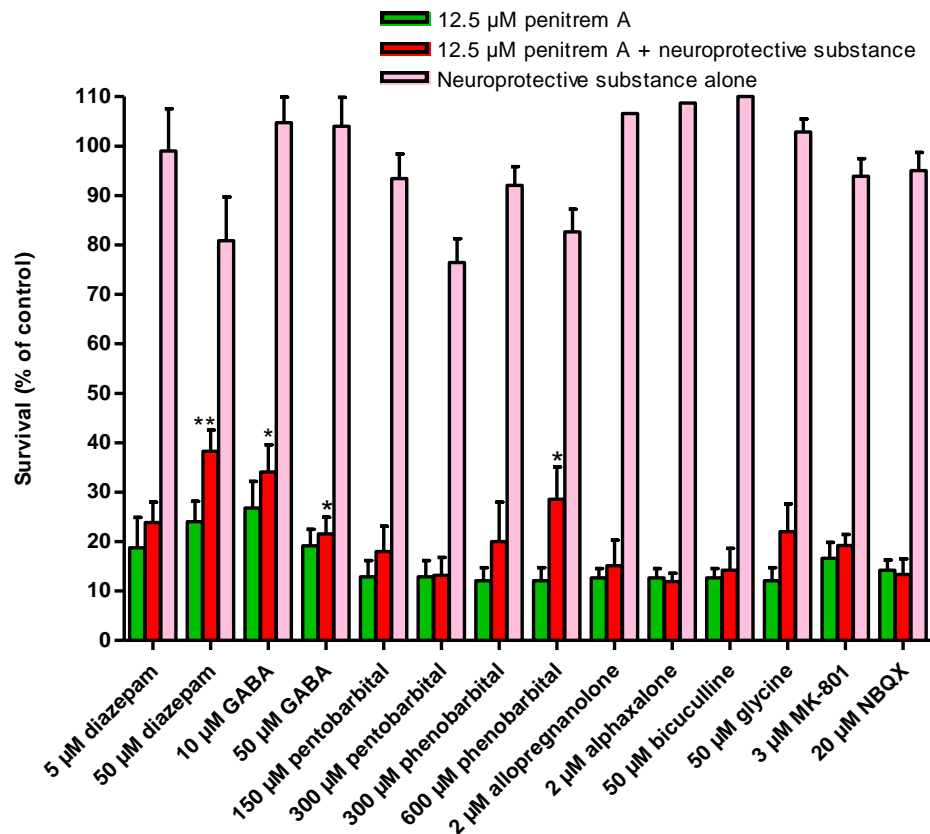


Figure 3-8. Survival of cerebellar granule cells in vitro after 24 hours of exposure to 12.5 μM penitrem A and potentially neuroprotective agents primarily acting extracellularly. Survival was measured with the MTT survival assay. Mean survival of cells + SE after exposure to 12.5 μM penitrem A alone and in conjunction with neuroprotective agents is shown as percentage of control ($n = 3-8$ in triplicate). Differences in survival was analysed with a two-tailed, paired Student's *t*-test. The symbols *, **, and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Cells were also exposed to neuroprotective agents alone ($n = 3-6$ in triplicate, except $n = 2$ for 50 μM bicuculline). None of the neuroprotective agents significantly affected survival compared to control. A Welch test with a Games-Howell post hoc test was used. Values for 2 μM allopregnanolone and alphaxalone ($n = 1$) are included for completeness, but could not be tested statistically.

L-NAME: After incubation with 300 μM L-NAME, there was a small but significant increase in average survival of 3 (95 % CI [1, 6], $p = 0.0267$) percentage points compared to penitrem A alone ($n = 5$).

CsA: Compared to exposure to penitrem A alone, incubation with 0.5 μM CsA caused a significant increase in survival of 12 (95 % CI [6, 18], $p = 0.0032$) percentage points ($n = 7$).

Vitamin E: 50 μM vitamin E showed a significant increase in survival of 38 (95 % CI, [23, 53], $p = 0.0003$) percentage points when compared to toxin alone ($n = 10$).

SP600125: Compared to penitrem A alone, 10 μM SP600125 caused a significant increase in survival of 20 (95 % CI [11, 30], $p = 0.0016$) percentage points ($n = 8$).

FK-506: 1.5 μ M FK-506 caused a significant increase of 11 (95% CI [5, 16], $p = 0.0022$) percentage points compared to incubation with toxin alone ($n = 8$).

BAPTA-AM: Compared to penitrem A alone, incubation with 5 and 10 μ M BAPTA-AM caused increases in survival of 20 (95 % CI [7, 33], $p = 0.0086$) and 21 (95 % CI [9, 33], $p = 0.0056$) percentage points respectively ($n = 7-8$).

To test if survival was significantly different from control for cells incubated with neuroprotective agents alone, a Welch test with a Games-Howell post hoc test was used. Only 10 μ M SP600125 caused a statistically significant decrease in survival compared to control of 52 (95 % CI [33, 71], $p < 0.001$) percentage points.

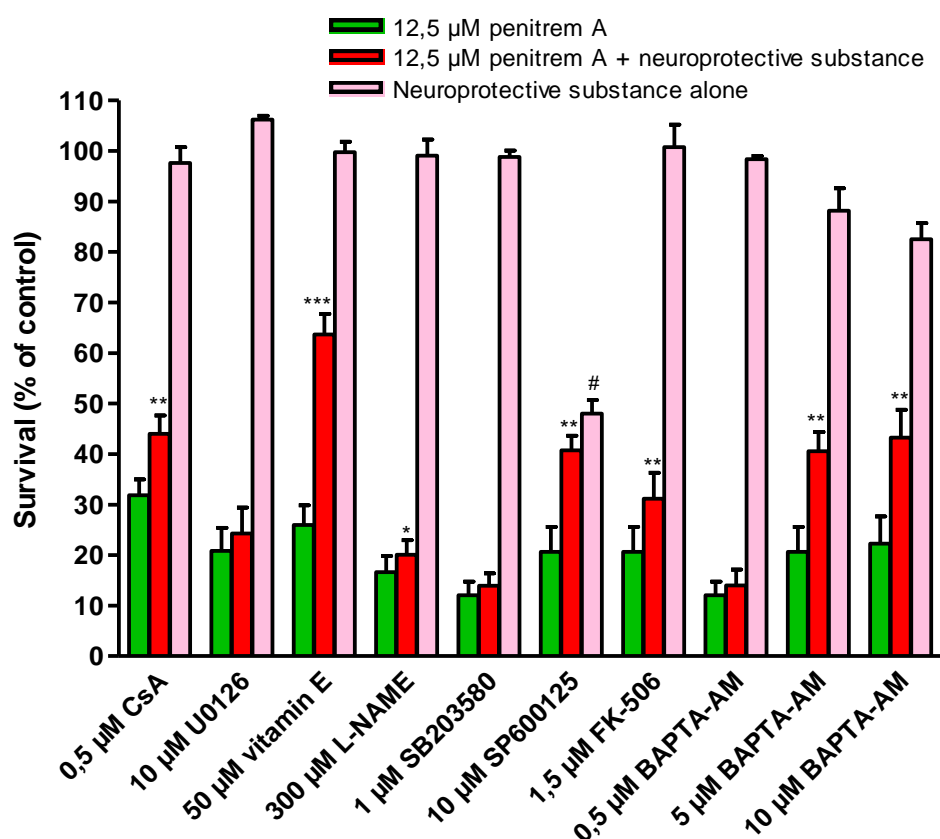


Figure 3-9. Survival of cerebellar granule cells in vitro after 24 hours of exposure to 12.5 μ M penitrem A and potentially neuroprotective agents primarily acting extracellularly. Survival was measured with the MTT survival assay. Mean survival of cells + SE after exposure to 12.5 μ M penitrem A alone and in conjunction with neuroprotective agents is shown as percentage of control ($n = 5-10$ in triplicate). Differences in survival was analysed with a two-tailed, paired Student's t -test. The symbols *, **, and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Cells were also exposed to neuroprotective agents alone ($n = 3-6$ in triplicate, except $n = 2$ for 0.5 μ M BAPTA-AM). To indicate where incubation with neuroprotective agents alone gave significantly different survival compared to control, the symbol # is used for $p < 0.05$. A Welch test with a Games-Howell post hoc test was used.

3.1.7 Survival after different exposure times to penitrem A and penitrem A with vitamin E

Exposure of cells to 12.5 μ M penitrem A and penitrem A in conjunction with 50 μ M vitamin E was carried out for 2, 6, 8, 12 and 24 hours ($n = 3-4$). Results are presented in figure 3-10.

For 12.5 μ M penitrem A alone, there was a time dependent decrease in survival compared to control with increasing exposure time. At all incubation times tested, the decrease in survival was significantly different from control, with an average decrease of 12 (95 % CI [5,19], $p = 0.002$) percentage points for 2 hours, 15 (95 % CI [7, 22], $p = 0.001$) for 6 hours, 22 (95 % CI [10, 33], $p = 0.002$) for 8 hours, 53 (95 % CI [8, 98], $p = 0.031$) for 12 hours, and 80 (95 % CI [61, 99], $p < 0.001$) percentage points at 24 hours exposure. An ANOVA with Dunnet's post hoc test was used for testing data from 2, 6, 8 and 24 hours of incubation, a Welch test with a Games-Howell post hoc test for the data from 12 hours of incubation.

Survival at 2, 6 and 8 hours exposure to 12.5 μ M penitrem A was significantly different from survival at 24 hours ($p = 0.015$, $p = 0.020$ and $p = 0.017$, respectively). A Welch test with Games-Howell post hoc test was used.

After incubation of cells with 12.5 μ M penitrem A in conjunction with 50 μ M vitamin E, only survival values at 2 and 24 hours exposure were significantly different from control ($p = 0.007$ and $p = 0.028$, respectively). An ANOVA with a Tukey-Kramer post hoc test was used.

A two-tailed, paired Student's t-test showed no significant difference in survival between cells incubated with and without vitamin E at 2, 6 and 8 hours. However, at 12 and 24 hours, incubation with vitamin E caused a significant increase in survival compared to penitrem A alone of 40.1 (95 % CI [0.4,79.7], $p = 0.049$) and 58.2 (95 % CI [29.8,86.6], $p = 0.0073$) percentage points, respectively.

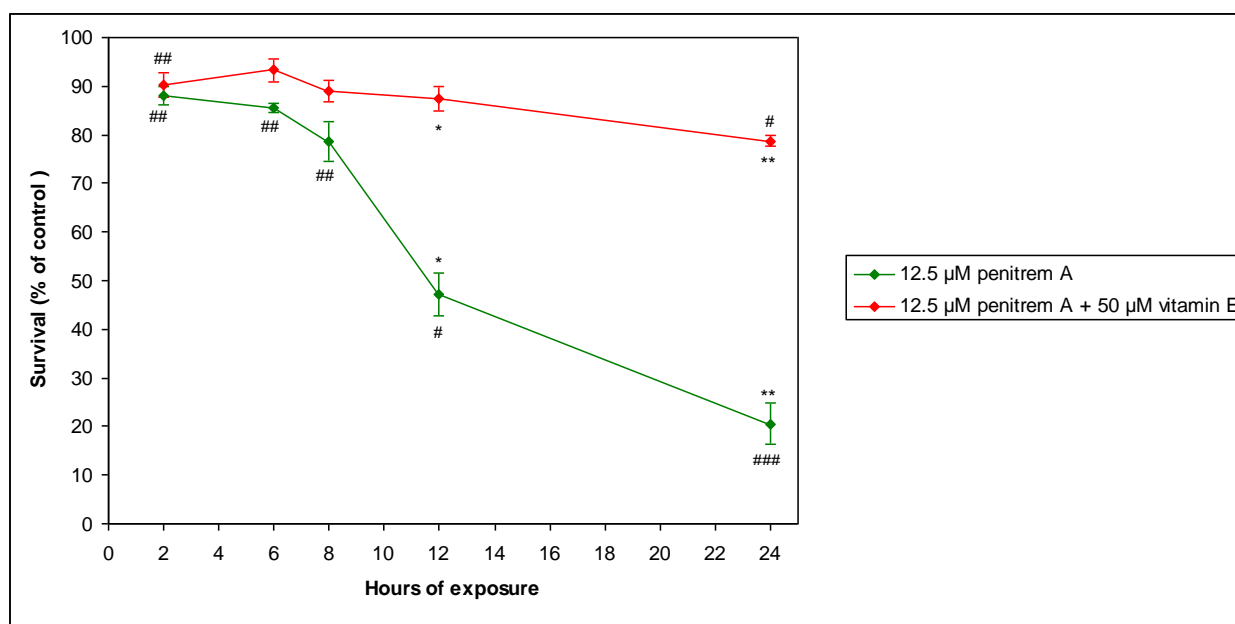


Figure 3-10. Survival of cerebellar granule cells *in vitro* after 2, 6, 8, 12 and 24 hours of exposure to 12.5 μ M penitrem A alone (green graph) and penitrem A in conjunction with 50 μ M vitamin E (red graph). Mean survival \pm SE is presented as percentage of control ($n = 3-4$ in triplicate). Survival values were measured with the MTT survival assay. Comparisons between control and 12.5 μ M penitrem A alone with and without 50 μ M vitamin E, were performed using an ANOVA and Dunnet's post hoc tests for 2, 6, 8 and 24 hours, and a Welch test with a Games-Howell post hoc test for 12 hours. The symbols # and ## indicate $p < 0.05$ and $p < 0.01$ respectively. A two-tailed, paired Student's *t*-test was used to compare 12.5 μ M penitrem A with and without inhibitor for each exposure time. Significant differences in survival with and without vitamin E are indicated by * and ** indicating p -values of $p < 0.05$ and $p < 0.01$ respectively.

3.2 ROS formation in human neutrophil granulocytes

3.2.1 ROS formation after PCB exposure measured with the DCF assay

For all the three PCBs, ROS production was found to be significantly different from control at 2.5, 5, 10 and 20 μ M ($p < 0.05$ for 2.5 and 5 μ M PCB 180, $p < 0.01$ for all others).

However, only for PCB 52 there was a significant increase in ROS production with increasing concentration for the doses tested. A Welch test with a Games-Howell post hoc test was used for PCB 52, whereas an ANOVA with a Dunnet's and Tuckey Kramer post hoc tests was used for PCB 153 and PCB 180 ($n = 6-9$). Average relative fluorescence values for the different PCBs are presented in figure 3-11.

Equal concentrations of the three PCBs were also compared using ANOVA and Tukey-Kramer tests, except for 5 μM , where a Welch test with a Games-Howell post hoc test was used.

2.5 μM : At 2.5 μM there was no significant difference in ROS production for the different PCBs.

5 μM : There was a significant difference in ROS production between 5 μM PCB 52 and PCB 180. 5 μM PCB 52 induced 43 (95% CI [8, 67], $p = 0.016$) % more ROS than 5 μM PCB 180. There was no significant difference between 5 μM PCB 52 and PCB 153 or between 5 μM PCB 153 and PCB 180.

10 μM : At 10 μM there was found to be a significant difference in ROS production between 10 μM PCB 52 and PCB 153 and between 10 μM PCB 52 and PCB 180. 10 μM PCB 52 induced 22 (95% CI [1, 59], $p < 0.05$) % more ROS than 10 μM PCB 153 and 41 (95% CI [12, 79], $p < 0.01$) % more than PCB 180. There was no significant difference between 10 μM PCB 153 and 10 μM PCB 180.

20 μM : Finally, there was a significant difference in ROS production between 20 μM PCB 52 and 20 μM PCB 180. 20 μM PCB 52 induced 40 (95% CI [9, 82], $p < 0.05$) % more ROS than 20 μM PCB 180. There was no significant difference between 20 μM PCB 52 and 153 or between 20 μM PCB 153 and 180.

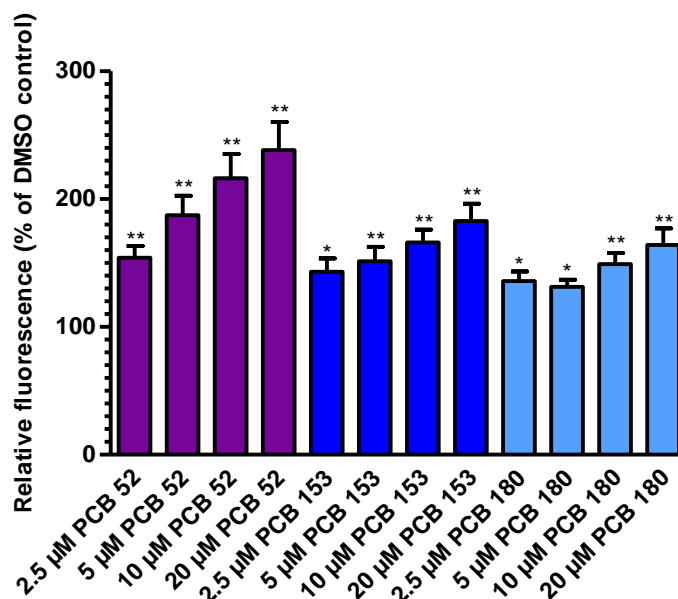


Figure 3-11. ROS production in human neutrophil granulocytes after exposure to 2.5, 5, 10, and 20 μ M of PCB 52, 153 and 180. ROS formation was registered using the DCF assay. Values were calculated as AUC relative to DMSO control after 2 hours of readings. Mean values and SE are displayed ($n=6-9$ in triplicate). Values that were significantly different from DMSO control after an ANOVA with a Dunnet's post hoc test have been marked with * for $p < 0.05$ or ** for $p < 0.01$.

3.2.2 DCF assay measurements of ROS production after PCB exposure in conjunction with potential ROS inhibitors

20 μ M PCB was tested in conjunction with a range of potential inhibitors of ROS formation. The inhibitors were also tested alone. The results for the different PCBs with and without each inhibitor, as well as for the inhibitors alone, are displayed in figure 3-12, 3-13 and 3-14 respectively.

In the text, in certain cases, the absolute decrease in relative fluorescence values in percentage points after addition of an inhibitor is presented in addition to the value for the decrease in percent. This has been done in the cases where a decrease has been found statistically significant, but where the value for the decrease in relative fluorescence for the PCB with inhibitor is very close to the value for the decrease in relative fluorescence in control incubated with an inhibitor alone. In these cases, it may be debated whether ROS inhibition in PCB/inhibitor combinations represent a genuine inhibition of the PCB-induced ROS production, or could merely be attributed to a decrease in basal ROS levels caused by the inhibitor. This is further commented upon in the discussion section.

Differences between ROS production in cells exposed to 20 μ M PCB with and without inhibitors were analysed with a two-tailed, paired Student's t-test. Inhibitors alone were compared to DMSO control using an ANOVA and a Dunnet's post hoc test.

CsA: In comparison with PCBs alone, 0.5 μ M CsA ($n = 6-7$) reduced ROS production significantly after exposure in conjunction with 20 μ M PCB 180, but not in conjunction with PCB 52 or PCB 153. 20 μ M PCB 180 with CsA caused 20 (95% CI [5, 34], $p = 0.017$) % less ROS production than PCB 180 alone. CsA alone was not significantly different from control ($n = 5$).

1 μ M U0126: When compared with PCBs alone, 1 μ M U0126 ($n = 7$) reduced ROS production significantly after exposure in conjunction with 20 μ M PCB 153 and PCB 180, but not in conjunction with PCB 52. For PCB 153, the decrease in fluorescence was 28 (95% [15, 41], $p = 0.0021$) percentage points, corresponding to a decrease in ROS production of 17 (95 % CI [9, 25]) %. For PCB 180 fluorescence was decreased with 41 (95% CI [21, 61], $p = 0.0027$) percentage points, which corresponds to a 28 (95 % CI [14, 41]) % decrease in ROS production. U0126 alone ($n = 7$) was significantly different from control, with a decrease in relative fluorescence of 28 (95 % CI [11, 45], $p < 0.01$) percentage points.

10 μ M U0126: 10 μ M U0126 ($n = 9$) caused a significant decrease in ROS production in conjunction with 20 μ M of all the three PCBs, when compared to PCBs alone. For PCB 52, the decrease was 66 (95 % CI [44, 88], $p = 0.0001$) %, for PCB 153 it was 70 (95 % CI [57, 82], $p < 0.0001$) %, and 75 (95 % CI [63, 87], $p < 0.0001$) % for PCB 180. 10 μ M U0126 alone ($n = 8$) was significantly different from control, with a decrease in relative fluorescence of 79 (95 % CI [63, 96], $p < 0.01$) percentage points.

Vitamin E: In comparison with PCBs alone, 50 μ M vitamin E ($n = 7-8$) reduced ROS production significantly in combination with 20 μ M PCB 52 and PCB180, but not with PCB 153. The decrease in ROS production was 35 (95 % CI [13, 58], $p = 0.0080$) % for PCB 52, and 13 (95 % CI, [2, 24], $p = 0.030$) % for PCB 180. 50 μ M vitamin E alone ($n = 7$) was not significantly different from control.

FK-506: When compared with PCBs alone, 1.5 μ M FK-506 ($n = 9$) reduced ROS production significantly in conjunction with 20 μ M PCB 180, but not with PCB 52 or 153. For PCB 180, the decrease in relative fluorescence was 30 (95 % CI, [20,40], $p < 0.0001$) percentage

points, corresponding to a decrease in ROS production of 21 (95 % CI [14, 28]) %. 1.5 μ M FK-506 alone (n = 9) was significantly different from control, with a decrease in relative fluorescence of 45 (95 % CI [30, 61], $p < 0.01$) percentage points.

SB203580: 1 μ M SB203580 (n = 9) caused a significant decrease of ROS production in conjunction with 20 μ M PCB 180 when compared to PCBs alone, whereas PCB 52 or 153 did not. The decrease in relative fluorescence for PCB 180 was 22 (95 % CI [13, 32], $p = 0.0007$) percentage points, which corresponds to a decrease in ROS production of 15 (95 % CI [9, 23]) %. 1 μ M SB203580 alone (n = 9) was significantly different from control, with a decrease in relative fluorescence of 18 (95 % CI [2, 34], $p < 0.05$) percentage points.

SP600125: When compared to PCBs alone, 10 μ M SP600125 (n = 9) reduced ROS production in conjunction with 20 μ M PCB 153 and PCB 180, but not with PCB 52. For PCB 153, the decrease in relative fluorescence was 56 (95 % CI [32, 80], $p < 0.0007$) percentage points, corresponding to 33 (95 % CI [19, 47]) % less ROS production than for PCB alone. For PCB 180, the decrease in fluorescence was 49 (95 % CI [39, 58], $p < 0.0001$) percentage points, corresponding to a 34 (95 % CI [27, 41]) % decreased ROS production. 10 μ M SP600125 alone (n = 9) was significantly different from control, with a decrease in relative fluorescence of 61 (95 % CI [45, 76], $p < 0.01$) percentage points

BAPTA-AM: In comparison with PCBs alone, 5 μ M BAPTA-AM (n = 8) in conjunction with 20 μ M of all the three PCBs caused significantly less ROS production. The decrease in ROS production was 32 (95 % CI [22, 42], $p = 0.0001$) % for PCB 52, 33 (95 % CI [19, 47], $p = 0.0007$) % for PCB 153, and 32 (95 % CI [25, 39], $p < 0.0001$) % for PCB 180. 5 μ M BAPTA-AM alone (n = 8) was significantly different from control, with a decrease in relative fluorescence of 24 (95 % CI [8, 40], $p < 0.01$) percentage points.

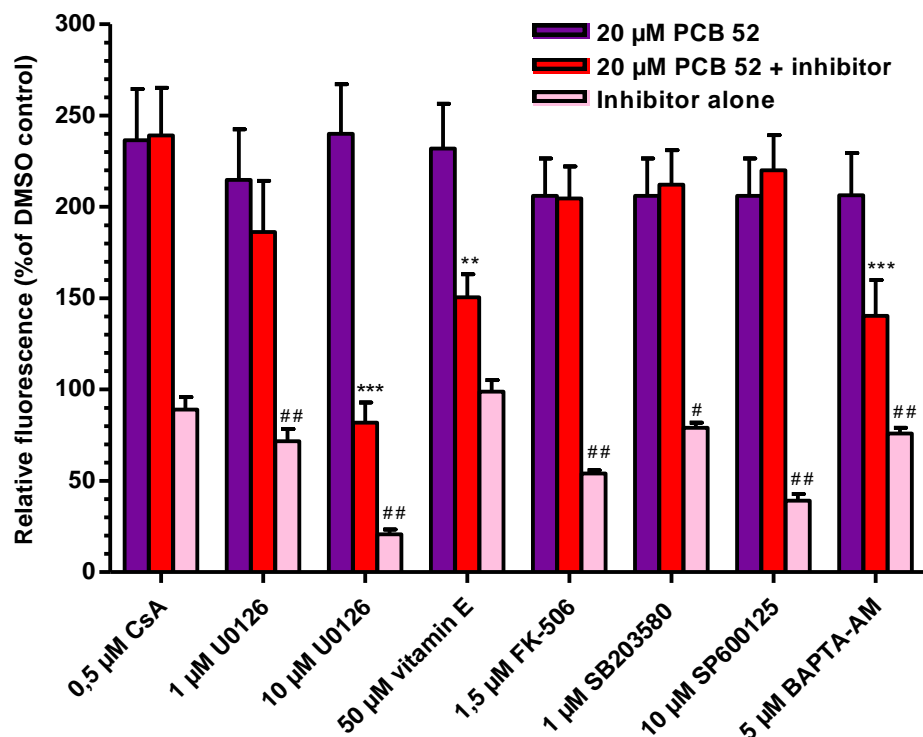


Figure 3-12. ROS production in human neutrophil granulocytes after exposure to 20 µM PCB 52 and different potential inhibitors of ROS. ROS production was measured using the DCF assay. Values were calculated as AUC relative to DMSO control after 2 hours of readings. Mean values and SE are given for 20 µM PCB 52 alone and in conjunction with an inhibitor ($n = 7-9$ in triplicate). Differences in ROS production were analysed with a two-tailed, paired Student's *t*-test. The symbols *, **, and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Cells were also exposed to inhibitors alone ($n = 7-9$ in triplicate). Inhibitors alone were compared to control with an ANOVA and a Dunnett's post hoc test. Significant differences from control are marked with # for $p < 0.05$ and ## for $p < 0.01$.

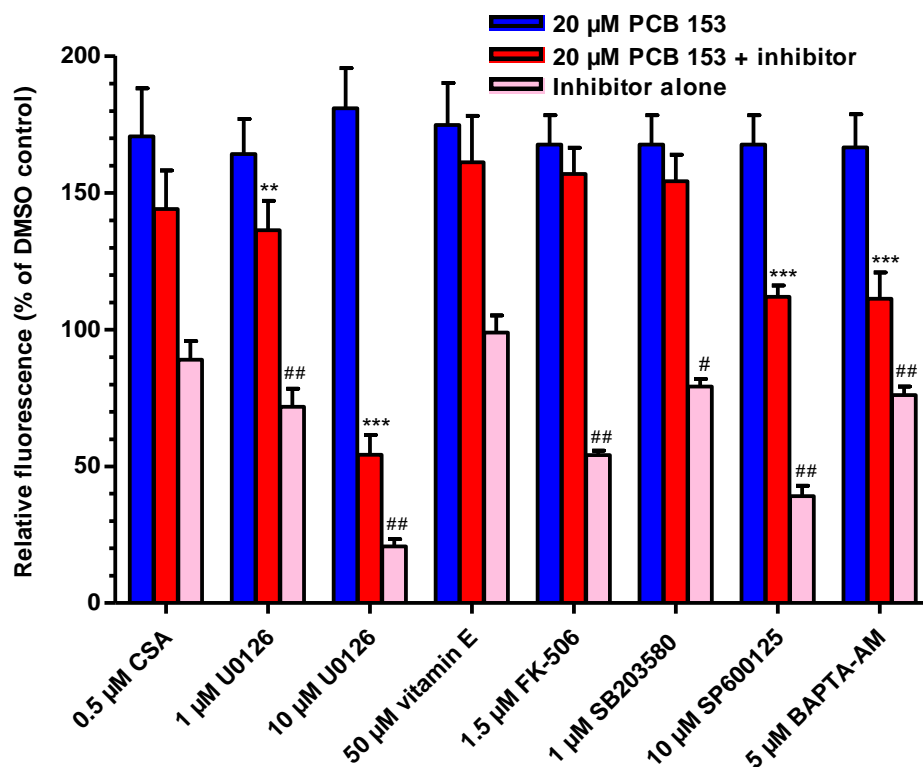


Figure 3-13. ROS production in human neutrophil granulocytes after exposure to 20 µM PCB 153 and different potential inhibitors of ROS. ROS production was measured using the DCF assay. Values were calculated as AUC relative to DMSO control after 2 hours of readings. Mean values and SE are given for 20 µM PCB 153 alone and in conjunction with an inhibitor ($n = 7-9$ in triplicate). Differences in ROS production were analysed with a two-tailed, paired Student's *t*-test. The symbols *, **, and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Cells were also exposed to inhibitors alone ($n = 7-9$ in triplicate). Inhibitors alone were compared to control with an ANOVA and a Dunnet's post hoc test. Significant differences from control are marked with # for $p < 0.05$ and ## for $p < 0.01$.

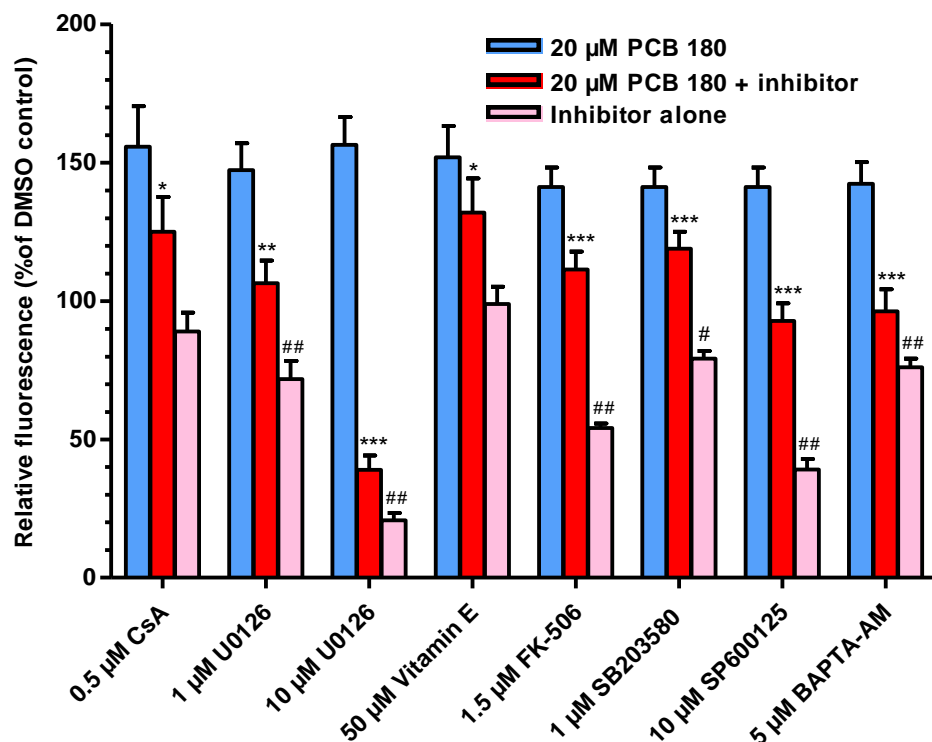


Figure 3-14. ROS production in human neutrophil granulocytes after exposure to 20 µM PCB 180 and different potential inhibitors of ROS. ROS production was measured using the DCF assay. Values were calculated as AUC relative to DMSO control after 2 hours of readings. Mean values and SE are given for 20 µM PCB 180 alone and in conjunction with an inhibitor ($n = 6-9$ in triplicate). Differences in ROS production were analysed with a two-tailed, paired Student's *t*-test. The symbols *, **, and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Cells were also exposed to inhibitors alone ($n = 7-9$ in triplicate). Inhibitors alone were compared to control with an ANOVA and a Dunnett's post hoc test. Significant differences from control are marked with # for $p < 0.05$ and ## for $p < 0.01$.

3.2.3 Luminol-chemiluminescence assay measurements of ROS production after exposure to PCBs

ROS production was also measured using the luminol-chemiluminescence assay after exposure of human granulocytes to 2.5, 5, 10 and 20 μM PCB 52, PCB 153 and PCB 180 ($n = 5-8$). Average relative chemiluminescence values for the different PCBs are presented in figure 3-15.

There was a dose dependent increase in ROS production with increasing concentration for all of the three PCBs. All the concentrations tested were significantly different from control for PCB 52 ($p = 0.019$ for 2.5 μM and 5 μM , $p = 0.013$ for 10 μM and $p = 0.001$ for 20 μM) and PCB 153 ($p = 0.006$ for 2.5 μM , $p = 0.023$ for 5 μM , $p = 0.016$ for 10 μM , $p = 0.014$ for 20 μM), whereas for PCB 180, this was the case for 10 and 20 μM ($p = 0.033$ and $p = 0.004$, respectively). An individual Welch test with a Games-Howell post hoc test was used for each PCB.

Equal concentrations of the three PCBs were also compared using ANOVA and Tukey-Kramer tests, except for 10 μM , where a Welch test with a Games-Howell post hoc test was used.

2.5 μM and 5 μM : At 2.5 and 5 μM there was no significant difference in relative luminescence for the different PCBs.

10 μM : At 10 μM there was a significant difference in ROS production when PCB 52 was compared to PCB 153 and PCB 180. PCB 52 caused 116 (95 % CI [3,232], $p < 0.049$) % more ROS production than 153, and 203 (95% CI [41,366], $p < 0.020$) % more than PCB 180. There was no significant difference in relative luminescence between 10 μM PCB 153 and 10 μM PCB 180.

20 μM : 20 μM PCB 52 also caused significantly more ROS production than PCB 153 and PCB 180. It caused 111 (95 % CI [37,186], $p < 0.01$) % more ROS production than PCB 153, and 138 (95 % CI [51,225], $p < 0.01$) % more than PCB 180.

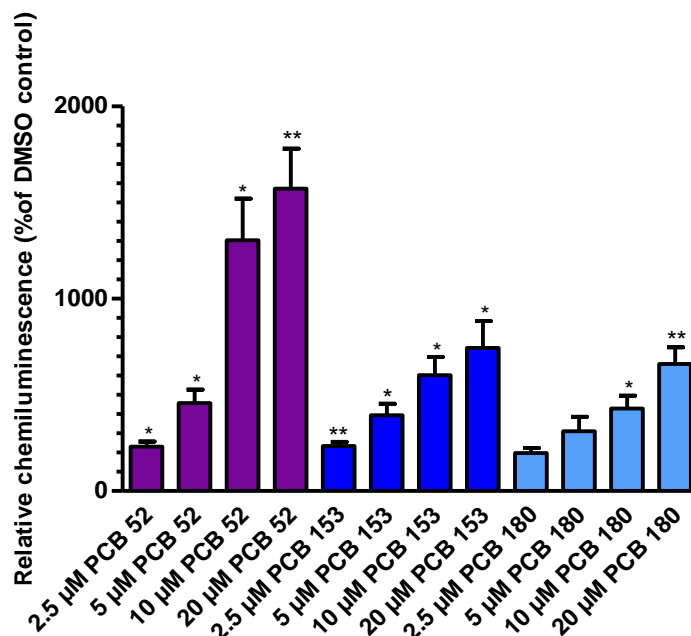


Figure 3-15. ROS production in human neutrophil granulocytes after exposure to 2.5, 5, 10, and 20 μM of PCB 52, 153 and 180. ROS production was measured using the luminol chemiluminescence assay. Values were calculated as AUC relative to DMSO control after 1 hour of readings. Mean values and SE are displayed ($n = 5-8$ in triplicate). Values that were significantly different from DMSO control after a Welch test with a Games-Howell post hoc test have been marked with * for $p < 0.05$ or ** for $p < 0.01$.

3.2.4 Luminol assay measurements of ROS production after exposure to PCBs in conjunction with ROS inhibitors

Also the luminol-chemiluminescence assay was used to measure ROS production for 20 μM PCB tested in conjunction with a range of potential inhibitors of ROS formation. The inhibitors were also tested alone. The results for the different PCBs with and without each inhibitor, as well as for the inhibitors alone, are displayed in figure 3-16, 3-17 and 3-18 respectively.

Differences between cells exposed to 20 μM PCB with and without inhibitors was analyzed by a two-tailed, paired Student's t-test. Inhibitors alone were compared to DMSO control by a Welch test with a Games-Howell post hoc test.

CsA: When compared to PCBs alone, 0.5 μM CsA ($n = 10-11$) reduced ROS production significantly in conjunction with 20 μM of all the three PCBs. The decrease in relative luminescence was 20 (95 % CI [11, 29], $p = 0.0006$) % for PCB 52, 23 (95 % CI [8, 38], $p = 0.0066$) % for PCB 153, and 25 (95 % CI [3, 47], $p = 0.0296$) % for PCB 180. CsA alone ($n = 10$) was significantly different from control, with a decrease in ROS production of 57 (95 % CI [24, 90], $p < 0.001$) %.

1 μM U0126: Compared to PCBs alone, addition of 1 μM U0126 ($n = 7-8$) reduced ROS production significantly in conjunction with 20 μM PCB 52, but not in conjunction with 20 μM PCB 153 or 20 μM PCB 180. The decrease in relative luminescence was 27 (95 % CI [6, 48] $p = 0.0212$) % for PCB 52. 1 μM U0126 alone ($n = 8$) was not significantly different from control.

10 μM U0126: 10 μM U0126 ($n = 9$) reduced ROS production significantly when in conjunction with 20 μM of all the three PCBs, compared to PCBs alone. The decrease in ROS production was 73 (95 % CI [49, 96], $p < 0.0001$) % for PCB 52, 69 (95 % CI [44,95], $p = 0.0003$) % for PCB 153, and 75 (95 % CI [54,97] $p < 0.0001$) % for PCB 180. 10 μM U0126 alone ($n = 9$) was significantly different from control, with a decrease in ROS production of 69 (95 % CI [42, 97], $p < 0.001$) %.

Vitamin E: Compared to PCBs alone, 50 μM vitamin E ($n = 9-11$) reduced ROS production significantly in conjunction with 20 μM of all the three PCBs. The decrease was 65 (95 % CI [42, 87], $p = 0.0002$) % for PCB 52, whereas it was 34 (95 % CI [18,48], $p = 0.0006$) % for PCB 153 and 38 (95 % CI [15,61], $p = 0.0049$) % for PCB 180. Vitamin E alone ($n = 10$) was not significantly different from control.

FK-506: There was a significant decrease in ROS production for all the three PCBs when incubated with 1.5 μM FK-506 ($n = 9-10$). The decrease was 42 (95 % CI [20,64], $p = 0.0023$) % for PCB 52, 34 (95 % CI [16,51], $p = 0.0017$) % for PCB 153, and 39 (95 % CI [20,58], $p = 0.0015$) % for PCB 180. FK-506 alone ($n = 9$), was significantly different from control, with a decrease in ROS production of 68 (95 % CI [37,98], $p < 0.001$) %.

SB203580: For all three PCBs, incubation of 20 μM PCB with 1 μM SB203580 ($n = 9-10$) reduced ROS production significantly compared to exposure to 20 μM PCB alone. The decrease was 41 (95 % CI [17, 65], $p = 0.0044$) % for PCB 52, 28 (95 % CI [7, 49], $p =$

0.0154) % for PCB 153 and 29 (95 % CI [7,50], $p = 0.0138$) % for PCB 180. SB203580 alone ($n = 9$) was significantly different from control, with a decrease in ROS production of 35 (95 % CI [10, 61], $p = 0.004$) %.

SP600125: 1 μ M SP600125 in conjunction with 20 μ M of all the three PCBs ($n = 9-10$) showed significantly lower ROS production than PCBs alone. The decrease was 44 (95 % CI [20, 68], $p = 0.0028$) % for PCB 52, 54 (95 % CI [24, 84], $p = 0.0026$) % for PCB 153 and 62 (95 % CI [32,92], $p = 0.0015$) % for PCB 180. SP600125 alone ($n = 9$) was significantly different from control, with a decrease in ROS production of 84 (95 % CI [50,118], $p < 0,001$) %.

BAPTA-AM: Finally, compared to PCBs alone, 5 μ M BAPTA-AM ($n = 9-10$) reduced ROS production significantly when incubated in conjunction with 20 μ M of all the three PCBs. The decrease in ROS production was 86 (95 % CI [46,126], $p = 0.0012$) % for PCB 52, 76 (95 % CI [47,105], $p = 0.0002$) % for PCB 153, and 68 (95 % CI [43, 93], $p = 0.0003$) % for PCB 180. BAPTA-AM alone ($n = 9$) was significantly different from control, showing a decrease in ROS production of 62 (95 % CI [22,102], $p = 0.002$) %.

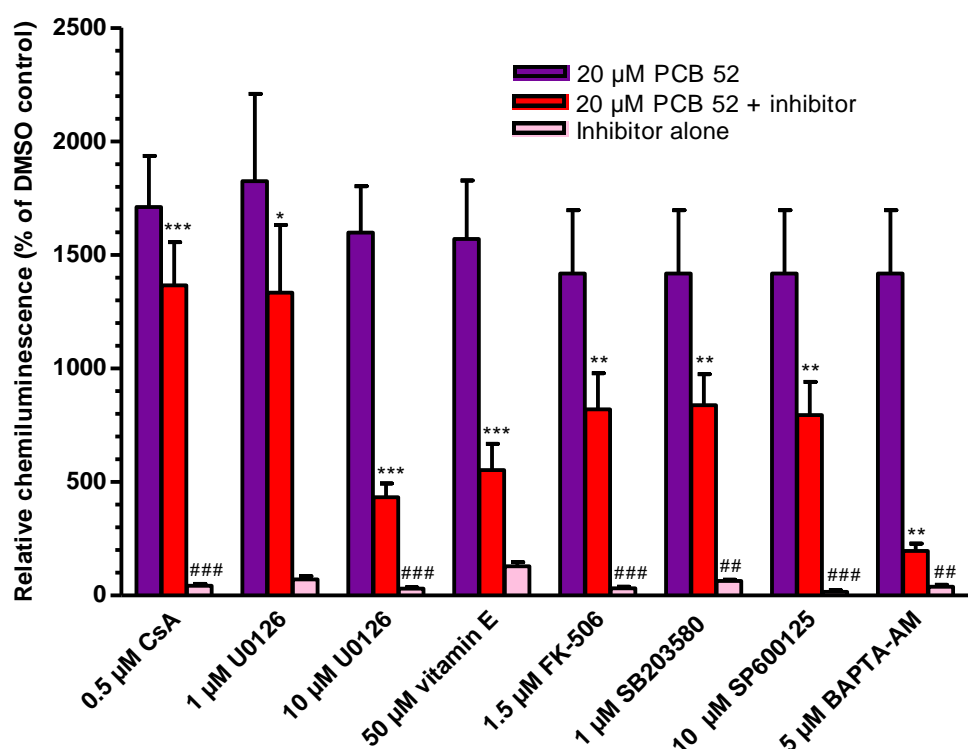


Figure 3-16. ROS production in human neutrophil granulocytes after exposure to 20 μM PCB 52 and different potential inhibitors of ROS. ROS production was measured using the luminol chemiluminescence assay. Values were calculated as AUC relative to DMSO control (set to 100 %) after 1 hour of readings. Mean values and SE are displayed ($n = 7-10$ in triplicate). Differences in ROS production were analysed with a two-tailed, paired Student's *t*-test. The symbols *, **, and *** indicate significance levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Cells were also exposed to inhibitors alone ($n = 8-10$ in triplicate). Inhibitors alone were compared to control with a Welch test and a Games-Howell post hoc test. Statistically significant differences between inhibitors alone and control are marked with # for $p < 0.05$, ## for $p < 0.01$ and ### for $p < 0.001$.

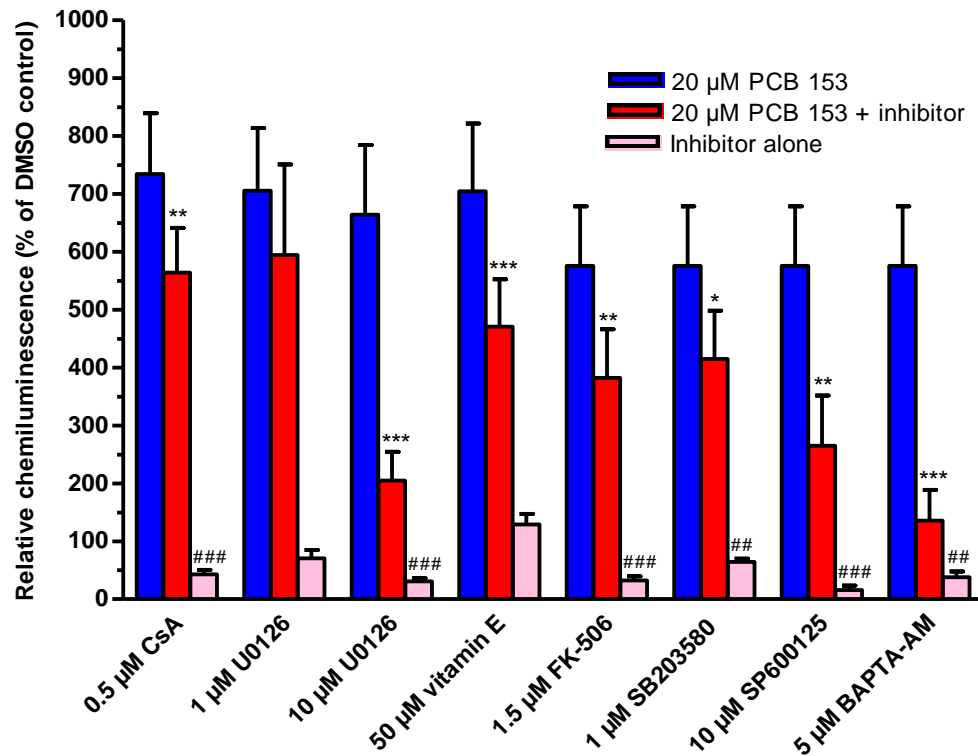


Figure 3-17. ROS production in human neutrophil granulocytes after exposure to 20 μ M PCB 153 and different potential inhibitors of ROS. ROS production was measured using the luminol chemiluminescence assay. Values were calculated as AUC relative to DMSO control after 1 hour of readings. Mean values and SE are displayed ($n = 8-11$ in triplicate). Differences in ROS production were analysed with a two-tailed, paired Student's *t*-test. The symbols *, **, and *** indicate significance levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Cells were also exposed to inhibitors alone ($n = 8-10$ in triplicate). Inhibitors alone were compared to control with a Welch test and a Games-Howell post hoc test. Statistically significant differences between inhibitors alone and control are marked with # for $p < 0.05$, ## for $p < 0.01$ and ### for $p < 0.001$.

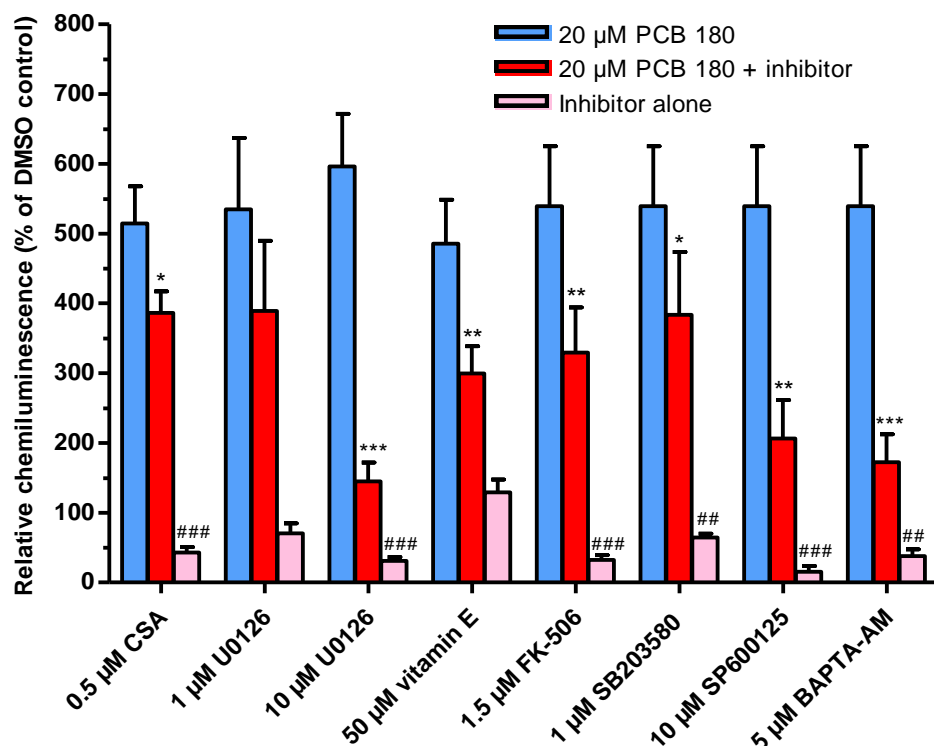


Figure 3-18. ROS production in human neutrophil granulocytes after exposure to 20 µM PCB 180 and different potential inhibitors of ROS. ROS production was measured using the luminol chemiluminescence assay. Values were calculated as AUC relative to DMSO control (set to 100 %) after 1 hour of readings. Mean values and SE are displayed ($n = 7-11$ in triplicate). Differences in ROS production were analysed with a two-tailed, paired Student's *t*-test. The symbols *, **, and *** indicate significance levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Cells were also exposed to inhibitors alone ($n = 8-10$ in triplicate). Inhibitors alone were compared to control with a Welch test and a Games-Howell post hoc test. Statistically significant differences between inhibitors alone and control are marked with # for $p < 0.05$, ## for $p < 0.01$ and ### for $p < 0.001$.

3.2.5 Preliminary studies of penitrem A-induced ROS production

Preliminary studies of ROS induction in human neutrophil granulocytes were conducted for 10 µM penitrem A together with PCB in fluorescence and chemiluminescence experiments. Results were compared using 20 µM 153 PCB as a positive control, as this PCB has previously been studied and shown to induce ROS formation (Reistad and Mariussen, 2005). Comparisons between groups were done using a Welch test and a Games-Howell post hoc test. Results are presented in figures 3-19 and 3-20.

With the DCF assay ($n = 9$) both 20 µM PCB 153 and 10 µM penitrem A reached values for ROS formation that were significantly different from the DMSO control. The relative fluorescence values after exposure to 20 µM PCB 153 was 166 ± 14 % (mean \pm SE) ($p = 0.003$), compared to 314 ± 24 % (mean \pm SE) ($p < 0.001$) for 10 µM penitrem A.

10 μ M penitrem A induced significantly more ROS than 20 μ M PCB 153. Penitrem A caused 89 (95 % CI [42,137], $p = 0.001$) % more ROS formation than the PCB. Because penitrem A induced significantly more ROS than the PCB, which was known to induce ROS production, it was decided that no positive control would have to be included in the further penitrem A ROS experiment.

With the luminol chemiluminescence assay ($n = 11$) exposure to 10 μ M penitrem A did not cause ROS levels that were significantly different from DMSO control. The relative chemiluminescence after exposure to 20 μ M PCB 153 was 669 ± 116 % (mean \pm SE) ($p = 0.002$), whilst 10 μ M penitrem A caused a chemiluminescence of 128 ± 16 % (mean \pm SE) ($p = 0.268$). The PCB 153 was interpreted as a positive control, indicating that penitrem A did not induce significant amounts of ROS that were detectable with the luminol assay.

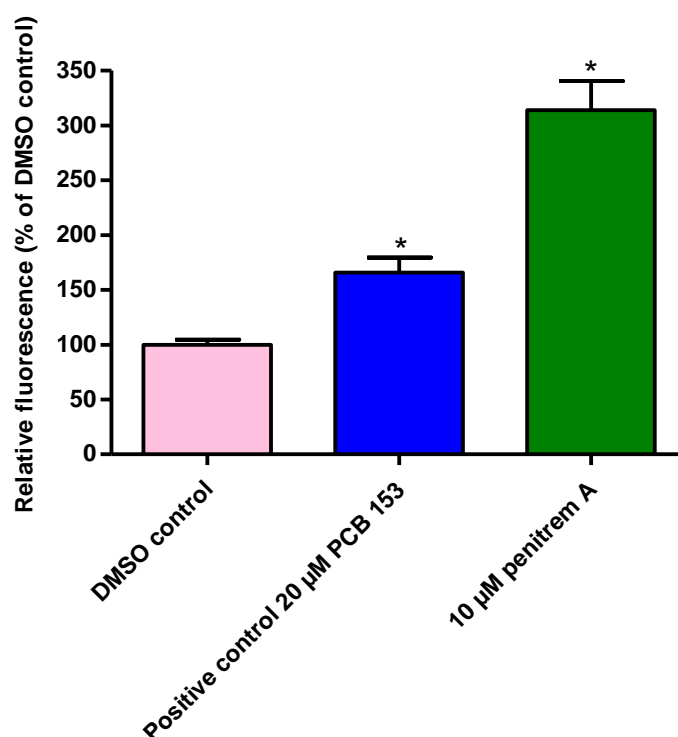


Figure 3-19. Relative fluorescence as a measure of ROS production in human neutrophil granulocytes after exposure to 10 μ M penitrem A, with 20 μ M PCB 153 as a positive control. Mean and SE for 9 experiments are shown. Values significantly different from control have been indicated with * representing a p -value of < 0.05 , assessed with a Welch test and a Games-Howell post hoc test.

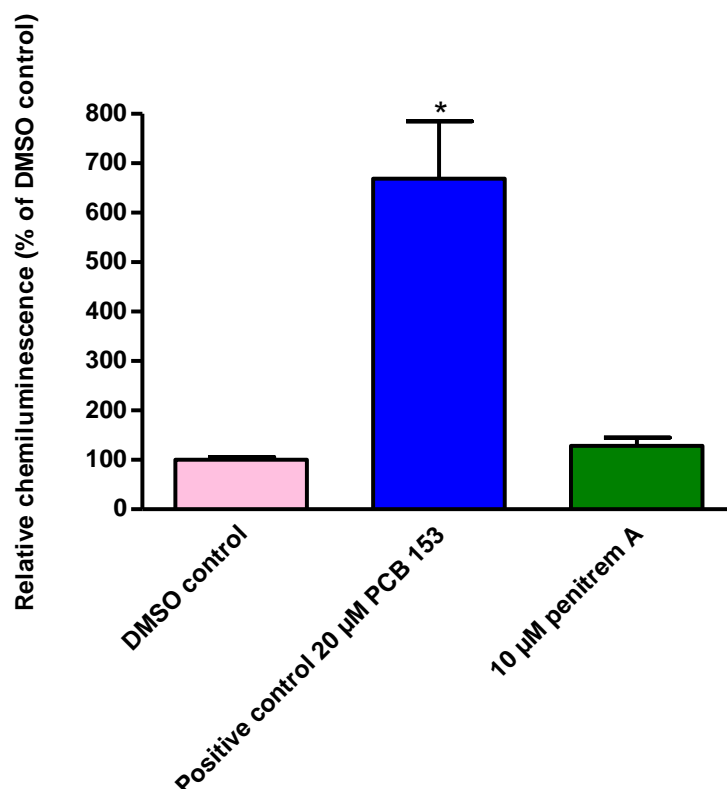


Figure 3-20. Relative chemiluminescence as a measure of ROS production in human neutrophil granulocytes after exposure to 10 µM penitrem A, with 20 µM PCB 153 as a positive control. Mean and SE for 11 experiments are shown. Values significantly different from control have been indicated with * representing a p -value of < 0.05 , assessed with a Welch test and a Games-Howell post hoc test.

3.2.6 Measurements of penitrem A-induced ROS production using the DCF assay

Also ROS production after exposure of human granulocytes to 1 nM, 10 nM, 250 nM, 750 nM, 2 µM, 10 µM and 12.5 µM penitrem A was measured using the DCF assay ($n = 5-9$). Average relative fluorescence values for penitrem A are presented in figure 3-21. For penitrem A, there was a dose dependent increase in ROS production with increasing concentration. Concentrations of 250 nM and above were significantly different from control when assessed with a Welch test and a Games-Howell post hoc test ($p = 0.017$ for 250 nM, $p = 0.029$ for 750 nM, $p = 0.005$ for 2 µM, $p = 0.014$ for 10 µM and $p = 0.035$ for 12.5 µM).

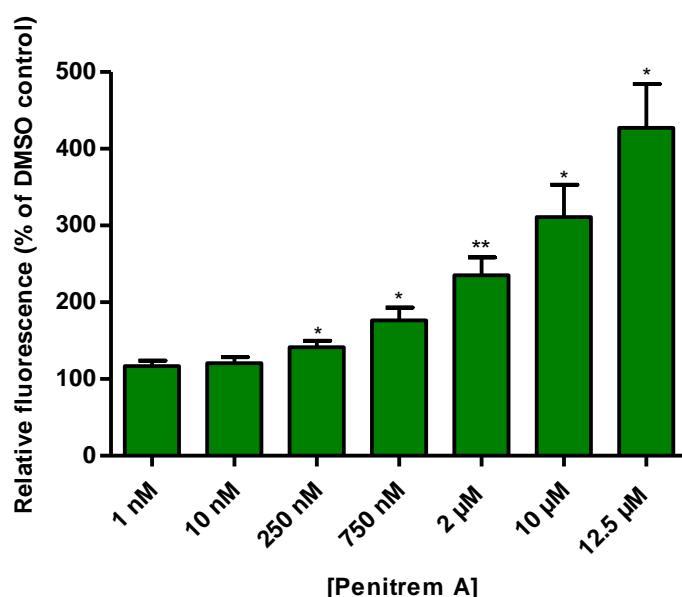


Figure 3-21. Relative fluorescence as a measure of ROS production in human neutrophil granulocytes, after exposure to 1 nM, 10 nM, 250 nM, 750 nM, 2 μ M, 10 μ M and 12.5 μ M penitrem A. Values were calculated as AUC relative to DMSO control after 2 hours of readings. Mean values and SE are displayed ($n = 5 - 9$). Values significantly different from DMSO control are indicated with * or **, representing $p < 0.05$ and $p < 0.01$, respectively. A Welch test and a Games-Howell post hoc test were used.

3.2.7 Potential inhibitors and their effect on ROS production after penitrem A exposure, assessed with the DCF-fluorescence assay

10 μ M penitrem A was analysed in the DCF assay in conjunction with several pharmacological inhibitors of intracellular pathways potentially leading to ROS formation. The inhibitors were also tested alone. Relative fluorescence values as a measure of granulocyte ROS production are displayed in figure 3-22. Differences in ROS production between cells exposed to 10 μ M penitrem A alone and in conjunction with an inhibitor were analyzed using a two-tailed, paired Student's t-test. Inhibitors alone were compared to DMSO control with an ANOVA and a Dunnet's post hoc test.

As discussed in chapter 3.2.2, also here absolute fluorescence values are given when found necessary, as these are relevant for the discussion of whether ROS inhibition in penitrem A/inhibitor combinations represent a genuine inhibition of the penitrem A-induced ROS production, or could merely be attributed to decreased basal ROS levels caused by the

inhibitor.

All the inhibitors tested caused a statistically significant decrease in ROS production with the exception of 1 μ M U0126.

CsA: For 0.5 μ M CsA ($n = 8$), there was a decrease in ROS production of 41 (95 % CI [21, 60], $p = 0.0017$) %. CsA alone ($n = 7$), was significantly different from the DMSO control, with a decrease in relative fluorescence of 24 (95 % CI [1, 48], $p < 0.05$) percentage points.

10 μ M U0126: For 10 μ M U0126 ($n = 7$), there was a decrease in ROS production of 51 (95 % CI [24, 78], $p = 0.0037$) %. 10 μ M U0126 alone ($n = 6$) was significantly different from the DMSO control, with a decrease in relative fluorescence of 72 (95 % CI [48, 96], $p < 0.01$) percentage points.

Vitamin E: For 50 μ M vitamin E ($n = 7$), there was a decrease in ROS production of 61 (95 % CI [28, 94], $p = 0.0041$) %. Vitamin E alone ($n = 6$) was not significantly different from the DMSO control.

FK-506: For 1.5 μ M FK-506 ($n = 6$), there was a decrease in ROS production of 41 (95 % CI [25, 57], $p = 0.0011$) %. FK-506 alone ($n = 6$) was significantly different from the DMSO control, with a decrease in relative fluorescence of 32 (95 % CI [8, 57], $p < 0.01$) percentage points.

SB203580: For 1 μ M SB203580 ($n = 6$), there was a decrease in ROS production of 28 (95 % CI [6, 51], $p = 0.0238$) %. SB203580 alone ($n = 6$) was not significantly different from the DMSO control.

SP600125: For 1 μ M SP600125 ($n = 6$), there was a decrease in ROS production of 52 (95 % CI [36, 68], $p = 0.0004$) %. SP600125 alone ($n = 6$) was significantly different from the DMSO control, with a decrease in relative fluorescence of 62 (95 % CI [37, 86], $p < 0.01$) percentage points.

BAPTA-AM: For 5 μ M BAPTA-AM ($n = 6$), there was a decrease in ROS production of 32 (95 % CI [8, 56], $p = 0.0185$) %. BAPTA-AM alone ($n = 6$) was significantly different from the DMSO control, with a decrease in relative fluorescence of 26 (95 % CI [1, 50], $p < 0.05$) percentage points.

L-NAME: Finally, for 300 μ M L-NAME ($n = 6$), there was an average decrease in relative fluorescence of 25 (95 % CI [1, 48], $p = 0.0423$) percentage points, corresponding to a decrease of 8 (95 % CI [0.3, 15]) %. L-NAME alone ($n = 6$) was not significantly different from the DMSO control, though it had a non significant decrease in relative fluorescence of 23 (95 % CI [-2, 47], $p > 0.05$) percentage points.

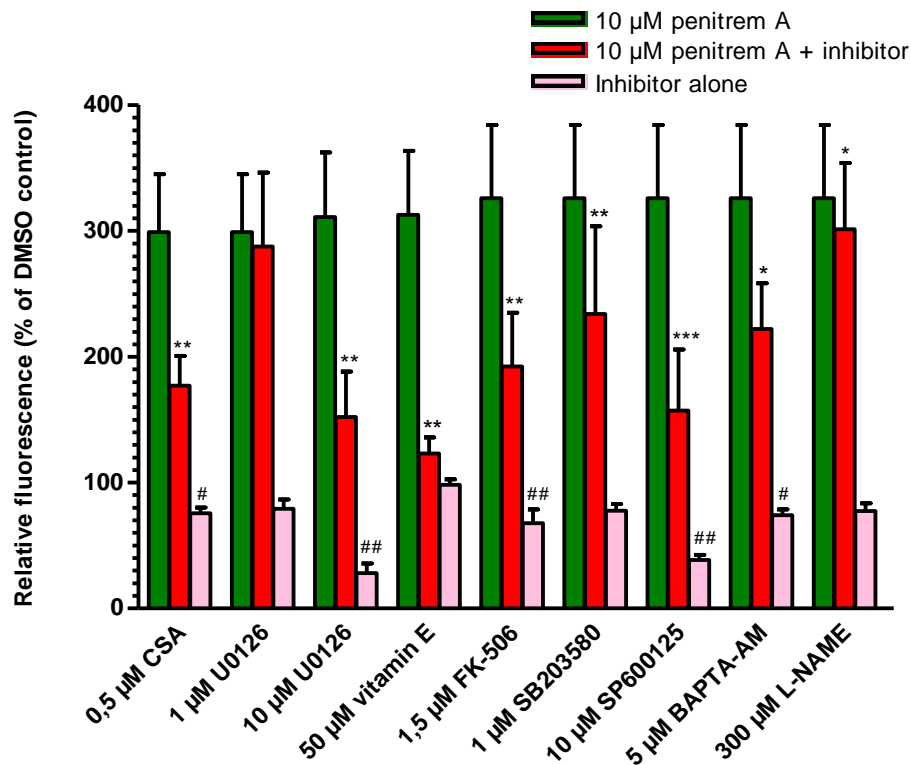


Figure 3-22. ROS production in human neutrophil granulocytes after exposure to 10 μ M penitrem A and different potential inhibitors of ROS. ROS production was measured using the DCF assay. Values were calculated as AUC relative to DMSO control after 2 hours of readings. Mean values and SE are given for 10 μ M penitrem A alone and in conjunction with an inhibitor ($n = 6-8$ in triplicate). Differences in ROS production were analysed with a two-tailed, paired Student's *t*-test. The symbols *, **, and *** indicate significance levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Cells were also exposed to inhibitors alone ($n = 6-7$ in triplicate). Inhibitors alone were compared to control with an ANOVA and a Dunnet's post hoc test. Significant differences from control are marked with # for $p < 0.05$ and ## for $p < 0.01$.

3.3 LDH assay

The LDH assay was carried out to assess that no significant damage to the cell membrane of the human granulocytes had occurred during incubation with the toxic substances. No statistically significantly elevated levels of LDH were detected for 20 μ M PCB 52, 153 or 180 or for 10 μ M penitrem A, indicating that the cell membranes had not been disrupted during the incubation period. An ANOVA with a Dunnett's post hoc test was used for statistical comparisons.

4. Discussion

4.1 Main results

In this study higher chlorinated PCBs induced more cell death in cerebellar granule cell cultures than lower chlorinated congeners. Cytoprotective effects were found for the substances vitamin E, MK-801 and U0126. The mycotoxin penitrem A caused more cell death in the same cell type with increasing concentration and exposure time. The substances GABA, phenobarbital, diazepam, vitamin E, BAPTA-AM, SP600125, FK-506 and CsA reduced cell death after penitrem A exposure.

The lower chlorinated PCBs induced more ROS production in human neutrophil granulocytes than the higher chlorinated congeners. The substances U0126, BAPTA-AM, vitamin E, SP600125, SB203580, FK-506 and CsA significantly reduced ROS production after PCB exposure. There was a dose dependent increase in ROS production in the granulocytes with exposure to increasing concentrations of penitrem A. The same substances inhibited ROS production after penitrem A exposure as after PCB exposure.

4.2 Cerebellar granule cells

4.2.1 Cerebellar granule cells as a model

Cerebellar granule cells were used to study the neurotoxicity of different PCBs and the mycotoxin penitrem A. Results from studies in primary cell cultures may potentially vary a lot depending on the state of the cells. It was observed that several factors could influence the quality of the cells, such as the batch of calf serum and bovine serum albumin used. The time from decapitation of rat pups until the cerebellum was removed seemed to be important, as well as the total time used for the isolation of cells. This has also been described by Oberdoerster (2001).

The cell quality seemed to affect the PCB results by shifting the dose response curve horizontally. However, this seemed to affect the PCBs equally, and the relative differences between PCBs should not be influenced. Variation would also be observed between results from different laboratories. Results from toxicity studies obtained *in vitro* may also vary if different assays are used to estimate cell viability. A method such as the trypan blue assay, which detects a disrupted cell membrane, will not necessarily give the same result as the MTT assay, which detects viable mitochondria. Finally, results obtained from *in vitro* cultures may not correspond to result from *in vivo* studies. Cells may be influenced by other factors in cultures than in the body, and many factors present in the body are impossible to mimic *in vitro*. In any case, the values obtained *in vitro* can only represent a “best estimate”.

Furthermore, when the toxic substances were tested in conjunction with potentially neuroprotective drugs, it seemed important to do this at a concentration where the toxic substances alone did not reduce survival too much. If tested with a concentration that resulted in a very low level of survival, the neuroprotective substances seemed unable to influence cell death. Because of this, it was chosen to test both 15 and 20 μM solutions of PCB 180 with the neuroprotective substances, whereas PCB 52 was tested with the substances at 20 μM . 15 μM PCB 180 and 20 μM PCB 52 gave approximately the same level of survival.

For penitrem A, cell quality seemed to affect results to a lesser degree than for the PCBs.

Overall, cerebellar granule cells were found to be good models for neurotoxicity studies after exposure to both PCB and penitrem A.

4.2.2 Toxicity studies of low and highly chlorinated PCBs

In order to assess if there were differences between the toxicity of low- and highly chlorinated *ortho*-substituted PCBs, cerebellar granule cells were exposed to the tetrachlorobiphenyl PCB 52 and the heptachlorobiphenyl PCB 180. Both PCBs showed a dose dependent decrease in survival with increasing concentration. There was a statistically significant difference between the LC_{50} values at 24 hours for the two PCBs, estimated to 14.3 and 9.5 μM for PCB 52 and PCB 180, respectively, indicating a higher toxicity of the highly chlorinated PCB.

Using the trypan blue exclusion assay, Mariussen *et al.* (2002) estimated the LC₅₀ value for another highly chlorinated *ortho*-substituted PCB, the hexachlorobiphenyl PCB 153, to 8 µM after 24 hours of exposure. This is quite close to the LC₅₀ value estimated for PCB 180 during these studies, despite the fact that the results were obtained in different laboratories with different techniques.

Cerebellar granule cells were also exposed to a selection of *ortho*-substituted PCBs with increasing degree of chlorination. The figures 3-5 and 3-6 show that toxicity increased with increasing concentration and chlorination.

PCB 28, the lowest chlorinated PCB, was not significantly different from control at any concentration. PCB 28 is a mono-*ortho*-chlorinated trichlorobiphenyl that does not bind to the Ah receptor (Robertson and Hansen, 2001). Exposure of neonatal mice *in vivo* to PCB 28 has been associated with neurotoxic effects in the adult (Eriksson and Fredriksson, 1996). *In vitro* studies of acutely isolated cerebellar granule cells have shown neurotoxicity of PCB 28 (Tan *et al.*, 2004). One can not rule out that it would have exerted significant neurotoxic effects had higher doses been tested.

At the lowest concentration tested, PCB 101 did not cause a significant reduction in survival when compared to control, but did so for the higher concentrations. For the higher chlorinated PCBs, the pentachlorobiphenyl PCB 110, the hexachlorobiphenyl PCB 153 and the heptachlorobiphenyl PCB 180, caused a significant reduction in survival even at 10 µM, indicating a higher toxicity for these congeners than the trichlorobiphenyl PCB 28, the tetrachlorobiphenyl PCB 52 and the pentachlorobiphenyl PCB 101.

Higher toxicity of PCBs with higher chlorination was also reported by Tan *et al.* (2004), in a study of acutely isolated cerebellar granule cells, involving di-, tri- and tetrachlorobiphenyls, including PCB 28 and PCB 52. Mariussen *et al.* (2001) found that higher chlorinated *ortho*-substituted PCBs partially inhibited GABA and glutamate uptake in brain synaptosomes even at very low concentrations, which may lead to excitotoxicity (Mariussen *et al.*, 2001).

It has been reported that PCBs with high and low degrees of chlorination may act via different mechanisms. In an *in vitro* study by Fernandes *et al.* (2010), which included all the above mentioned PCBs except PCB 110, the lower chlorinated congeners PCB 28 and PCB 52 were shown to act as agonists at the GABA_A receptor. These effects are not seen for the

higher chlorinated PCBs. Furthermore, in an *in vivo* study by Boix *et al.* (2010), offspring from female rats showed different symptoms after exposure of the mother to low- and highly chlorinated PCBs during pregnancy and lactation. The lower chlorinated PCB, represented by PCB 52, showed a negative effect on motor coordination. The higher chlorinated PCBs, represented by PCB 138 and PCB 180, affected learning ability.

Boix *et al.* (2010) proposed that developmental exposure to PCB 52 increases GABA in the cerebellum, thus impairing motor coordination, whereas exposure to PCB 138 and 180 could cause a decrease in the amount of NMDA receptors in the cerebellum. GABA is involved both in learning, memory and motor activity (Fernandes *et al.*, 2010). Activation of NMDA receptors causes an increase in intracellular calcium, which activates NOS with a subsequent increase in NO. This again activates guanylate cyclase and increases cyclic GMP (cGMP). Decreased activation of this pathway due to a decreased number of NMDA receptors could cause impairment of learning abilities (Boix *et al.*, 2010).

The highly chlorinated PCB 153, which was found to be one of the most neurotoxic PCBs in this study, has often been the most prominent congener detected in breast milk, human tissue and blood (Guo *et al.*, 1997; Kowalski *et al.*, 2010). Epidemiological studies indicate that PCB exposure is associated with learning disabilities and hyperactivity disorders (Mariussen and Fonnum, 2006). Thus, it is a cause of concern that infants may be exposed to this neurotoxic congener via placental transfer and ingestion of breast milk from mothers with high levels of PCBs in their tissues.

To summarise, the presented results strongly indicate that higher chlorinated *ortho*-substituted PCBs are more toxic than the lower chlorinated PCBs to cerebellar granule cells *in vitro*. However, lower chlorinated PCBs may also exert a toxic effect. As postulated by Fernandes *et al.* (2010) and Boix *et al.* (2010), different mechanism may be involved in the toxicity of higher chlorinated and lower chlorinated PCBs.

4.2.3 Potential mechanisms of PCB neurotoxicity

Several substances were studied in conjunction with PCB 52 and 180 to assess whether any of these had a cytoprotective effect. Vitamin E, an antioxidant, increased cerebellar granule cell survival after exposure to both PCB 52 and PCB 180, indicating an involvement of ROS

production. Vitamin E has been included in previous studies of PCB toxicity in cerebellar granule cells. Mariussen *et al.* (2002) found that it protected against cell death, and also reduced ROS formation, indicating a connection between free radical formation and cell death. As earlier mentioned, vitamin E is thought to scavenge lipid peroxyl radicals (Wang and Quinn, 2000), singlet oxygen ($^1\text{O}_2$) (Kaiser *et al.*, 1990), ONOO^- (Hogg *et al.*, 1994) and $\text{O}_2^{\cdot-}$ (Ha and Csallany, 1992). Unpublished results from our laboratory show that ROS production was induced in cerebellar granule cells after exposure to several *ortho*-chlorinated PCBs, with an increase in ROS production related to decreasing chlorination (Dreiem A., personal communication, 2010). At 20 μM , the increase in survival after incubation with vitamin E was 42 percentage points in conjunction with PCB 52, as compared to 24 percentage points for PCB 180. The fact that the increase in survival with vitamin E was greater for the lower chlorinated PCB may reflect that it induces higher levels of ROS production than the higher chlorinated.

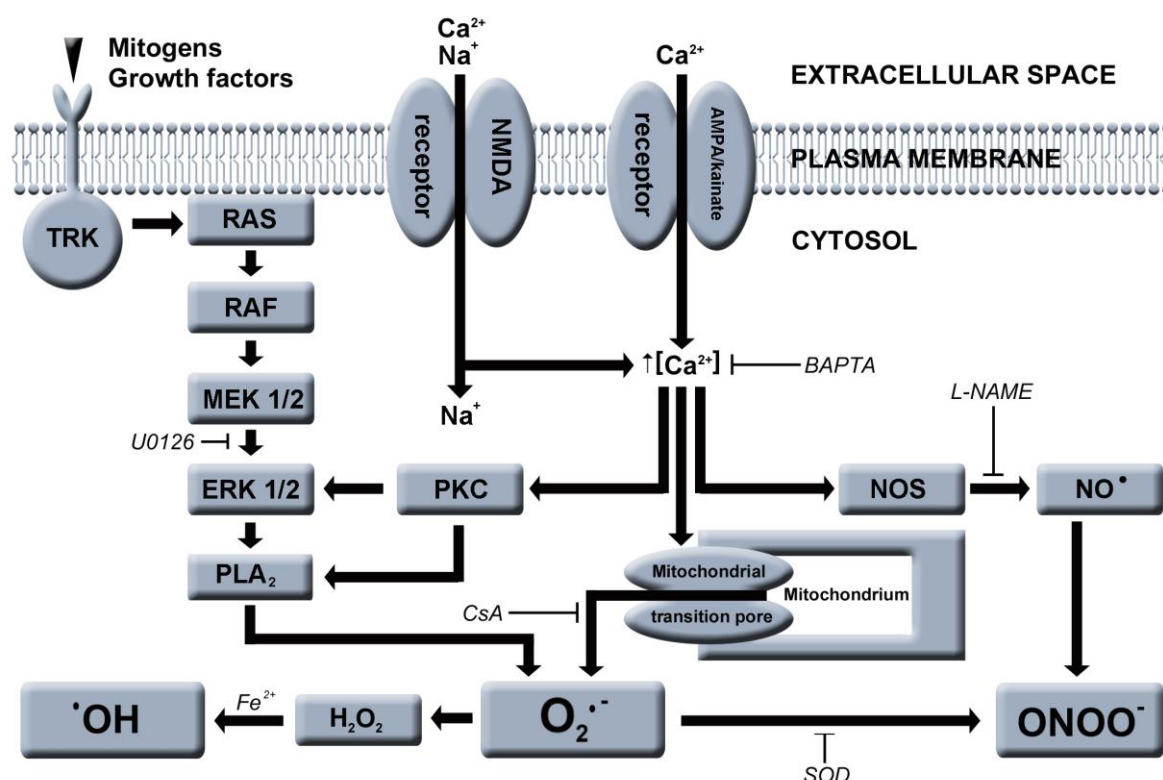


Figure 4-1. Pathways that may be activated by PCBs leading to ROS formation in cerebellar granule cells. Potential inhibitors of the pathways are shown in *italics*. Adapted from Fonnum *et al.* (2006).

MK-801 increased survival with by 17 percentage points for PCB 52 at 20 μM , and 19 percentage points at 15 μM PCB 180. This may indicate that the NMDA receptor is involved in the mechanism of toxicity for both PCBs. MK-801 binds inside the calcium channel of the

NMDA receptor, preventing influx of calcium ions into the cell. PCB is thought to bind to the NMDA receptors and increase the number of receptors on the cell surface, triggering an influx of calcium, which may lead to activation of several cascades leading to apoptosis or necrosis (Ndountse and Chan, 2009). Involvement of the NMDA receptor and protective effects of MK-801 after PCB exposure have previously been reported by Mariussen *et al.* (2002) in cerebellar granule cells, and by Ndountse and Chan (2009) in neuroblastoma cells.

Ndountse and Chan (2009) also found a protective effect of memantine after PCB exposure. Memantine is also an NMDA receptor blocker, but a less potent one than MK-801 (Weller *et al.*, 1993). In the current study no significant effect was found for 3 μ M memantine. Ndountse and Chan, however, used a higher dose of 10 μ M. In future studies, it may be of interest to use higher doses of memantine also in cerebellar granule cells.

BAPTA-AM, on the other hand, seemed to potentiate the toxic effect of 20 μ M PCB 52, causing a decrease in survival of 24 percentage points and a non-significant decrease of 27 percentage points for 15 μ M PCB 180 ($p = 0.054$). As the focus in this study main was on identifying substances that had protective properties, only 3 experiments were carried out with BAPTA-AM at 15 μ M PCB 180. Given the p -value and the magnitude of the decrease, it is possible to hypothesise that BAPTA-AM in conjunction with PCB 180 may also have exerted a negative effect on survival. This, however, cannot be confirmed without further studies.

The reduced survival observed using BAPTA-AM during PCB studies in cerebellar granule cells was in contrast to the increased survival observed in human neuroblastoma cells by Ndountse and Chan (2009). Their findings were done after exposure of neuroblastoma cells to BAPTA-AM in conjunction with PCB 126, PCB 99 and Araclor 1254. BAPTA-AM is an intracellular calcium chelator (Ndountse and Chan, 2009). The maintenance of intracellular calcium homeostasis is necessary for a normally functioning cell (Mariussen and Fonnum, 2006). Thus, decreasing the intracellular levels of free calcium may possibly interfere with the cell control mechanisms. Changes in intracellular calcium levels after PCB exposure may occur by different mechanisms in different types of cell preparations prior to cell death (Fonnum and Mariussen, 2009). This may possibly contribute to the diverging results from the two cell types. However, further research would be needed to make conclusions about this effect of PCBs and BAPTA-AM.

For cerebellar granule cells exposed to 15 μ M PCB 180, the MEK 1/2 and MEK 5 inhibitor U0126 also increased survival by 8 percentage points. The biological relevance of this could be questioned, but Dreiem *et al.* (2009) found that survival in cerebellar granule cells after exposure to hydroxylated PCBs was increased after addition of U0126. This was, however, to a much greater extent than observed here. CsA caused a trivial increase in survival of 2 percentage points. The biological relevance of this result, however, is probably negligible. U0126 and CsA did not cause any significant change in survival when incubated with PCB 52. L-NAME did not increase survival in any of the PCB experiments.

To summarise, the results indicate that both PCB 52 and PCB 180 seem to act via the NMDA receptor, and ROS production may be involved in their toxicity, as indicated by the protective effect of vitamin E. Disturbances of cellular calcium homeostasis seem to potentiate the toxicity of PCB 52.

4.2.4 Penitrem A neurotoxicity

To assess the neurotoxic potential of penitrem A in cerebellar granule cells, the cells were incubated with different concentrations of the toxin for 24 hours. A dose dependent decrease in survival after exposure was observed. The LC₅₀ value for penitrem A was estimated to 10.5 μ M. There have been no published results on penitrem A toxicity in cerebellar granule cells, but the presence of necrotic foci in the granular cell layer of the cerebellum were reported by Cavanagh *et al.* (1998), 6 hours after intraperitoneal injections of the toxin *in vivo*. In the same study, substantial degeneration of Purkinje cells, another type of neurone found in the cerebellum, was found.

The results show that penitrem A is toxic to cerebellar granule cells *in vitro*.

4.2.5 Possible mechanisms of penitrem A neurotoxicity

To elicit factors involved in penitrem A-induced death in cerebellar granular cells, the toxin was studied in conjunction with different substances in order to identify if any of these would increase survival after exposure to the toxin. There are currently two main theories concerning the cause of the neurological symptoms after exposure to fungal diterpenes such as penitrem A. One involves the blockage of high conductance calcium activated potassium

channels (BK channels) in the presynaptic neuronal membrane. This may lead to an increased duration of action potentials by the prevention of cell repolarisation, causing an increased release of neurotransmitters from the presynaptic membrane.

The other theory is that the neurological symptoms are caused by an imbalance between excitatory and inhibitory influences in the brain (Moldes-Anaya, A., 2010). Moldes-Anaya *et al.* (2010) found that penitrem A inhibited reuptake of GABA and glutamate in rat cerebellar synaptosomes. They postulated that this may cause an accumulation of GABA and glutamate in the synaptic cleft, prolonging the action of the neurotransmitters on their receptors.

Moldes-Anaya *et al.* (2010) also found that penitrem A did not bind to the NMDA or AMPA receptors, which opposes an earlier proposed hypothesis that penitrem A induces excitotoxicity via direct action on these receptors. It was also hypothesised that penitrem A binds to a site on the GABA_A receptor that partly overlaps with the binding site for the GABA antagonist bicuculline. Bicuculline is a convulsant that blocks synaptic inhibition through GABA receptors by antagonising the action of GABA at the GABA binding site (Johnston, 1996; Rang *et al.*, 2007).

In this study, several different drugs acting on the GABA_A, NMDA and AMPA receptors were tested. In concurrence with the findings of Moldes-Anaya *et al.* (2010), there was no significant increase in cerebellar granule cell survival after exposure to penitrem A when incubated in conjunction with the NMDA receptor antagonist MK-801 or the AMPA receptor antagonist NBQX. There was no significant increase in cell survival when penitrem A exposed cells were incubated in conjunction with glycine.

However, there was a 14 percentage point increase in survival after incubation with a 600 µM solution of the barbiturate phenobarbital, and a 17 percentage point increase after incubation with a 50 µM solution of the benzodiazepine diazepam. This indicates that the GABA_A receptor may be involved in mechanisms leading to cell death after penitrem A exposure. Alleviation of convulsions in dogs intoxicated with penitrem A after ingestion of contaminated food has been observed after treatment with pentobarbital and phenobarbital, both alone or in conjunction with the anticonvulsant diazepam (Moldes-Anaya, A., 2010).

There was also minor significant increases in survival of 7 and 2 percentage points after exposure to 10 and 50 µM GABA, respectively. The biological relevance of such small

increases, however, is questionable. There was no significant change in survival after exposure to penitrem A in conjunction with the GABA antagonist bicuculline, the neurosteroids allopregnanolone or alphaxolone, or the barbiturate pentobarbital.

Vitamin E significantly increased survival by 38 percentage points when cells were incubated with this in conjunction with penitrem A. This suggests a potential role of ROS production in the toxicity of penitrem A in nerve cells, as vitamin E is a fast acting antioxidant scavenging both lipid peroxy radicals and intracellular free radicals.

Furthermore, concentrations of 5 and 10 μ M of the intracellular calcium chelator BAPTA-AM caused increases of 20 and 21 percentage points, respectively, after incubation in conjunction with penitrem A. This suggests that an increase in intracellular free calcium is induced during penitrem A exposure. However, this should be confirmed with further studies.

The MAPKs belong to a family of protein kinases that transmit signals to the nucleus. As earlier mentioned, five subfamilies have been identified, the ERK 1/2, ERK 3/4, JNK, p38 and ERK 5 (Schaeffer and Weber, 1999; Chen *et al.*, 2005). The MAPKs are believed to be important molecules in cell death induced by extracellular stimuli (Schaeffer and Weber, 1999).

SP600125 is a selective inhibitor of the JNK pathway (Bennett *et al.*, 2001). FK-506 and CsA are both inhibitors of the JNK and p38 MAPK pathways (Matsuda *et al.*, 2000), whereas SB203580 is a selective inhibitor of the p38 pathway (Cuenda *et al.*, 1995). U0126 is an inhibitor of the ERK 1/2 and ERK 5 pathways (Favata *et al.*, 1998; Kamakura *et al.*, 1999). Cerebellar granule cell survival after penitrem A exposure was increased by 20 percentage points when incubated with SP600125, and 10 and 12 percentage points with FK-506 and CsA respectively. No increase in survival was seen for SB203580 or U0126.

Increased survival after exposure to SP600125, FK-506 and CsA, but not after exposure to SB203580, suggests an involvement of the JNK pathway in penitrem A induced neuronal cell death, but not of the p38 pathway. Incubation of cells with SP600125 alone, decreased survival by 52 %, something which could indicate that the JNK pathway is important in the regulation of survival in cerebellar granule cells.

CsA may also inhibit the mitochondrial transition pore (Bernardi *et al.*, 1994). Opening of this pore by increased intracellular calcium may cause the production of $O_2^{\bullet-}$ (Fonnum *et al.*, 2006). Calcium is also capable of activating the NOS pathway, causing the formation of NO^{\bullet} (Fonnum *et al.*, 2006). L-NAME, a NOS inhibitor (Moncada and Higgs, 1991; Anderson and Meyer, 1996), also caused a trivial increase in survival of 3 percentage points. The biological relevance of this is debatable.

To summarise, penitrem A induced cell death in cerebellar granule cells seems to involve the $GABA_A$ receptor. An increase in intracellular free calcium may also be involved. Increased intracellular calcium may again be linked to the formation of ROS. Increased survival after incubation with vitamin E may indicate involvement of ROS formation in penitrem neurotoxicity. Increases in calcium may open mitochondrial transition pores, which may result in production of $O_2^{\bullet-}$. CsA blocks these pores (Fonnum *et al.*, 2006). An increase in survival after CsA treatment indicates that opening of mitochondrial transition pores may be a possible step in ROS production. Finally, the pro-apoptotic JNK pathway may possibly be involved in penitrem A neurotoxicity. Activation of this pathway has been linked to increases in calcium in studies involving macrophages and other toxicants (Kim and Sharma, 2004). In future studies, it may be interesting to assess whether this may also be the case in cerebellar granule cells after penitrem A exposure.

4.2.6 Neurotoxicity of penitrem A at different times of exposure

Cerebellar granule cells were incubated with penitrem A for 2, 6, 8, 12 and 24 hours to assess effects of exposure time on its neurotoxic potential. For 12.5 μM penitrem A alone, there was a time dependent decrease in survival with increasing exposure time. When cells were exposed to penitrem A in conjunction with vitamin E such a decrease in survival was not observed. Even at 12 and 24 hours, when survival in cells exposed to penitrem A was as low as 47 and 20 percent, respectively, incubation with vitamin E increased survival significantly to 87 and 78 percent.

In summary, this indicates that the toxic effect of penitrem A in cerebellar granule cells increases with exposure time, and that vitamin E gives a substantial protection even after 24 hours of exposure.

4.3 Human neutrophil granulocytes

4.3.1 Human neutrophil granulocytes as a model

Human granulocytes were used as a model to study ROS production after exposure to PCBs and penitrem A. During the preliminary experiments, it was observed that DMSO inhibited basal ROS production in control cells quite substantially, especially when measured by the DCF method. As all the substances that were to be tested for effects on ROS production, as well as the toxic substances, were dissolved in DMSO, it became apparent that DMSO controls with the same volume of DMSO had to be included. This allowed for fluorescence and luminescence values to be calculated as percentage of these controls, correcting for the inhibitory effect of DMSO on ROS production.

For the PCBs both the DCF method and the luminol chemiluminescence method gave useful results, as has previously been observed by Myhre *et al.* (2003) and Myhre *et al.* (2009). In the mentioned studies similar levels of ROS production were detected by the two methods. In the current study, however, ROS levels were much higher when measured with the luminol chemiluminescence method than with the DCF method.

For penitrem A, no significant ROS production was detected when assessed with the luminol chemiluminescence method, possibly indicating that HOCl is not the main ROS induced by the toxin. The DCF method, however, was found useful, detecting significant levels of ROS compared to DMSO control.

That no significant release of LDH was detected after incubation of cells with the toxic substances, indicated that the cell membranes had not been disrupted during the incubation period. This suggests that measured fluorescence/luminescence would be due to ROS created intracellularly in viable cells.

4.3.2 ROS production in human neutrophil granulocytes after PCB exposure

In order to assess if there was a difference between low and highly chlorinated PCBs in their potential to induce ROS production, cells were exposed to different doses of the

tetrachlorinated PCB 52, the hexachlorinated PCB 153, and the heptachlorinated PCB 180. ROS production was quantified using the DCF and luminol assays.

With both assays, all the tested doses of the three PCBs caused statistically significant increases in ROS compared to control, except 2.5 and 5 μ M PCB 180 with the luminol assay. All PCBs caused a dose dependent increase in ROS production when assessed with the luminol assay. However, when measured using the DCF assay, there was a dose dependent increase in ROS production with increasing concentration only for PCB 52.

For both assays, the lower chlorinated PCB 52 caused significantly higher levels of ROS production than the higher chlorinated PCBs 153 and 180 at 10 and 20 μ M. The exception was PCB 153 at 20 μ M, which was not significantly different from PCB 52 when measured with the DCF assay. However, differences between ROS induction after exposure to higher and lower chlorinated PCBs were much larger when measured using the luminol assay.

These results indicate that the lower chlorinated PCBs such as PCB 52 may induce a greater production of ROS in human neutrophil granulocytes than the highly chlorinated PCBs such as PCB 180. The reactive oxygen species detected by these methods are ONOO^- , $\cdot\text{OH}$, H_2O_2 and HOCl (Myhre *et al.*, 2003). In concurrence, Voie *et al.* (2000) reported that induction of ROS was dependent on size of the congener. Congeners of larger size, corresponding to PCBs with high congener numbers and many chlorine substitutions, were reported to be less potent. The most active were reported to be the moderately sized congeners with several *ortho*-substitutions.

Interestingly, as earlier mentioned, unpublished results from our laboratory also showed the same tendency in cerebellar granule cells. Lower chlorinated PCBs seemed to induce more ROS than the higher chlorinated ones when assessed with the DCF assay (Dreiem A., personal communication, 2010).

That the lower chlorinated PCBs may induce a greater production of ROS in human neutrophil granulocytes, could perhaps implicate that the lower chlorinated PCBs have a greater capability of inadvertent activation of neutrophils and effects on the immune system. Voie *et al.* (1998) postulated that defects in neutrophil function after PCB exposure may render the host more susceptible to pathogen invasion, or initiate inappropriate activation of inflammatory responses, causing tissue damage.

4.3.3 Assessment of pathways involved in ROS production after PCB exposure

The NADPH oxidase of the human neutrophil granulocytes may be activated by several signalling pathways, that may be interdependent (Myhre *et al.*, 2009). To try to identify some of the pathways involved in the induction of ROS production after exposure to PCBs, several pharmacological inhibitors of intracellular pathways were used in conjunction with the toxic substances. ROS was measured both using the DCF and the luminol chemiluminescence assays.

When cells were exposed to 10 μ M U0126, a reduction of approximately 70 % in ROS production was observed with both assays for all the three PCBs. U0126 inhibits MEK 1/2 and MEK 5 (Favata *et al.*, 1998; Kamakura *et al.*, 1999). MEK 1/2 is found upstream of ERK 1/2 MAPK and MEK 5 upstream of ERK5. Both ERK 1/2 and ERK 5 phosphorylate and activate NADPH oxidase (Aam, 2007). Thus, the results indicate that these pathways may be involved in the production of ROS for all the three PCBs.

When measured with the luminol assay, 1 μ M U0126 in conjunction with the PCBs caused a significant reduction in PCB 52 induced ROS production of 27 %. Measured using the DCF assay, 1 μ M U0126 reduced ROS production significantly for the higher chlorinated congeners PCB 180 and PCB 153 when compared to PCB alone. However, the reduction in relative fluorescence after PCB 153 exposure in conjunction with 1 μ M U0126 was not larger than the decrease in relative fluorescence observed in cells exposed to 1 μ M U0126 alone (see figure 3-13). Due to this, one can not rule out that the decrease was only due to a inhibition of basal ROS production (see section 2.4.2) in the cell and not due to a decrease in the PCB induced ROS. Ultimately, this means that although the decrease in ROS production was statistically significant, it is not possible to conclude from the DCF data that there is a real effect of the inhibitor.

BAPTA-AM reduced ROS production in PCB exposed granulocytes for all PCBs with approximately 30 % and 68-86 % when measured with the DCF and luminol assays, respectively. As BAPTA-AM is an intracellular calcium chelator (Ndountse and Chan, 2009), this may indicate that increased intracellular calcium levels are involved in the induction of ROS production.

Incubation with vitamin E caused significant reduction in ROS production for all PCBs measured with both assays, with the exception of 20 μ M PCB 153 assessed with the DCF assay. Decreases were higher for the lower chlorinated PCB 52 than for the others. The observed reduction in ROS levels might be expected as an effect of vitamin E, as it is a known fast-acting antioxidant and ROS scavenger.

Several of the tested inhibitors act on the JNK and p38 MAPK pathways. SP600125 is a selective inhibitor of the JNK pathway (Bennett *et al.*, 2001), SB203580 a selective inhibitor of the p38 pathway (Cuenda *et al.*, 1995), and FK-506 and CsA may act on both pathways (Matsuda *et al.*, 2000). Measured with the luminol assay SP600125 decreased ROS production with 44-62 %, whereas SB203580 caused a reduction of 28-41 % for the three different PCBs. FK-506 decreased ROS production with 34-42 % and CsA 20-25 % for the three congeners. For PCB 180, CsA showed a similar decrease when measured with the DCF assay. All these results suggest the involvement of both the p38 MAPK and the JNK pathways in ROS production for all the different PCBs.

The problem of distinguishing between inhibition of basal ROS production and PCB-induced ROS production, as described for 1 μ M U0126, was also observed when ROS was measured using the DCF assay for some other inhibitors. These included the p38 and JNK pathway inhibitor FK-506, the selective p38 inhibitor SB203580 and the selective JNK inhibitor SP600125. The DCF data for these inhibitors did not allow for conclusions to be drawn about their effects, although they caused statistically significant decreases in ROS production. The data from the luminol data proved more useful in this respect.

Overall, the results suggest that similar pathways seem to be involved in inducing ROS production in human neutrophil granulocytes after exposure to the three different PCBs. Due to an observed reduction in ROS production caused by the different inhibitors, the four MAPK families ERK 1/2, JNK, p38 and ERK 5 are likely to be involved in induction of ROS production. An increase in intracellular calcium also seems important for the ROS production.

Previous findings have shown that single ortho-substituted PCBs and PCB mixtures such as Aroclor 1242 induce ROS formation in human neutrophil granulocytes (Voie *et al.*, 1998; Voie *et al.*, 2000; Myhre *et al.*, 2003; Myhre *et al.*, 2009). MAPK, PKC and Ca^{2+} are thought

to be important factors in the activation of NADPH oxidase (Myhre *et al.*, 2009). Voie *et al.* (1998) hypothesised that PCB activated ROS production in human granulocytes was dependent on the phosphatidylinositol-3 kinase, the Ca^{2+} dependent phospholipase D (PLD) and/or phospholipase C (PLC) and protein kinase C (PKC) activation before activation of the NADPH oxidase (Voie *et al.*, 1998). PLD may also be involved in activation of the p38 pathway (Myhre *et al.*, 2009). In addition, Myhre *et al.* (2009) found that the ERK 1/2 kinases, p38 MAPK, JNK and/or calcineurin pathways were involved in the activation of the respiratory burst after exposure to Aroclor 1242.

A presentation of the possible signalling pathways involved in the PCB induced activation of NADPH during ROS production, and inhibitors of the different pathways, is presented in figure 4-2.

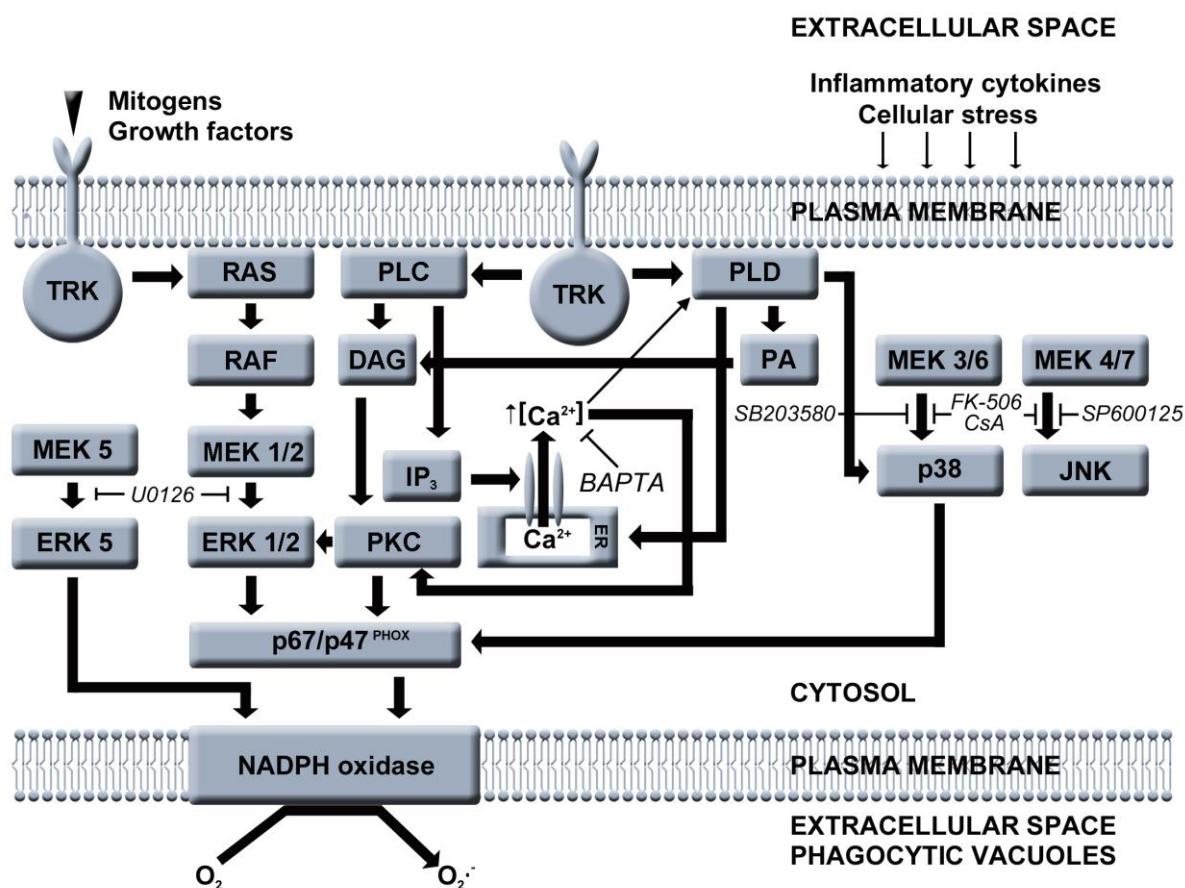


Figure 4-2. Important intracellular pathways involved in ROS formation in human neutrophil granulocytes after PCB exposure. The MAP kinase pathways ERK 1/2, ERK 5, p38 and JNK are shown. The ERK 1/2 pathway is activated through tyrosine kinases (TRK) by extracellular signal substances. P38 and JNK pathways are primarily activated in situations of cellular stress. The phospholipases C and D (PLC/PLD) may also activate protein kinase C via intermediate signal substances. IP_3 produced by phospholipases causes release of Ca^{2+} from the endoplasmic reticulum (ER). PLD may be involved in activation of the p38 pathway. Adapted from Myhre et al. (2009).

4.3.4 ROS production in human neutrophil granulocytes after penitrem A exposure

To assess the ability of penitrem A to induce ROS production, human neutrophil granulocytes were exposed to different concentrations of penitrem A. A dose dependent increase in ROS production was detected with increasing concentrations, and concentrations above 250 nM induced statistically significant levels of ROS production as compared to control. Thus the results indicate that ROS production may be an important mechanism of action in penitrem induced cell damage. An investigation on the ability of penitrem A to induce ROS production in nerve cells, would be an interesting target for further studies.

4.3.5 Mechanisms of ROS-induction after penitrem A exposure

To identify possible pathways involved in the penitrem A-induced ROS production, the effects of inhibitors of different intracellular pathways were assessed. Penitrem A-induced ROS production was significantly inhibited by the MEK 1/2 and MEK 5 inhibitor U0126, the p38 and JNK pathway inhibitors FK-506 and CsA, the selective p38 inhibitor SB203580 the selective JNK inhibitor SP600125, the intracellular calcium chelator BAPTA-AM and vitamin E. The NOS inhibitor L-NAME had only a minor effect.

The results thus suggest that the MAPK families ERK1/2, ERK 5, p38 and JNK may all be involved in the induction of ROS production in human neutrophil granulocytes after exposure to penitrem A. Increased levels of intracellular Ca^{2+} may also contribute to the increase in ROS formation.

5. Conclusions and future perspectives

One of the aims of this thesis was to investigate whether there are differences in neurotoxicity between low and highly chlorinated *ortho*-substituted PCBs, and attempt to identify factors involved in their neurotoxicity. Based on the results presented, there is a strong indication that the higher chlorinated *ortho*-substituted PCBs are more toxic to cerebellar granule cells than are the lower chlorinated. However, the latter are also neurotoxic. As discussed different mechanisms may be involved in the toxicity of lower and higher chlorinated PCBs, giving rise to distinct effects after *in vivo* exposure.

In the current study, ROS production and NMDA receptor involvement in neurotoxicity in cerebellar granule cells were indicated by the protective actions of the antioxidant vitamin E, and the NMDA receptor channel blocker MK-801 for both PCB 52 and 180. As mentioned, it was also observed in unpublished studies from our laboratory that a higher level of ROS production was induced by lower chlorinated PCBs than the higher chlorinated ones. This could possibly suggest that ROS production is more important in neurotoxicity induced by lower chlorinated PCBs than for the higher chlorinated PCBs.

An interesting aim for future studies could be experiments with specific inhibitors of ROS production pathways in cerebellar granule cells, using the same PCBs. Also, some of the inhibitors used in the human granulocyte experiment, such as FK-506, SP600125 and SB203580 could be tested in studies of PCB-induced ROS production in cerebellar granule cells. This might elicit if any of the same pathways that are involved in ROS production in the granulocytes also are involved in cell death in the cerebellar granule cells. Further replicates with the inhibitor BAPTA-AM could be performed in order to obtain statistically significant results also for PCB 180. However, the usefulness of performing further PCB toxicity studies in cerebellar granule cells must be weighted against the fact that the experiments require sacrifice of a considerable number of animals.

Results from human neutrophil granulocyte experiments showed that the lower chlorinated PCBs were more potent at inducing ROS production than the highly chlorinated PCBs at higher concentrations. Studies with inhibitors of different intracellular pathways indicated that the MEK 1/2 and MEK 5 pathways, the p38 pathway and the JNK pathway, as well as

an increase in intracellular calcium, may all be involved in the induction of ROS production in human neutrophil granulocytes after PCB exposure.

The results from neurotoxicity studies of penitrem A indicate that it is toxic to cerebellar granule cells *in vitro*. Studies with potentially neuroprotective substances indicated that the GABA_A receptor could be involved in the induction of cell death. An increase in intracellular calcium and the activation of the JNK pathway may also be involved. Increased survival after co-incubation of penitrem A with vitamin E indicated that ROS production could potentially be involved in the mechanism of toxicity.

The ability of penitrem A to induce ROS production was further confirmed by the human granulocyte experiments. The MEK 1/2, the MEK 5, the p38 and the JNK pathways as well as an increase in intracellular calcium could be involved in induction of ROS in human neutrophil granulocytes after penitrem A exposure.

In future studies, it would be interesting to use the DCF method to assess ROS production in cerebellar granule cells after penitrem A exposure, as this was the method found to yield interesting results in the human neutrophil granulocytes. Furthermore, it would be of interest to study intracellular levels of free calcium after penitrem A exposure, and also to assess if there is a link between intracellular calcium levels and ROS production, or between calcium levels and activation of the JNK pathway.

The exact mechanism of penitrem A neurotoxicity has not been identified. As mentioned, the studies of the mycotoxin showed that it was capable of inducing ROS production in human neutrophil granulocytes. ROS production in cerebellar granule cells was also suggested by the protective action of vitamin E. Based on the findings it could be hypothesised that ROS production could be an important cause of cell death after penitrem A exposure, in addition to the two earlier proposed mechanisms of an inactivation of BK channels or an imbalance between excitatory and inhibitory influences in the brain (Moldes-Anaya, A., 2010). Further studies on penitrem A-induced ROS production in nerve cells should be conducted.

6. References

- Anderson, R. E., F. B. Meyer (1996). "Nitric oxide synthase inhibition by L-NAME during repetitive focal cerebral ischemia in rabbits." Am J Physiol Heart Circ Physiol **271**: 588-594.
- Arkoosh, M. R., E. Clemons, M. Myers and E. Casillas (1994). "Suppression of B-cell mediated immunity in juvenile chinook salmon (*Oncorhynchus tshawytscha*) after exposure to either a polycyclic aromatic hydrocarbon or to polychlorinated biphenyls." Immunopharmacol Immunotoxicol **16**(2): 293-314.
- Beal, M. F. (1995). "Aging, energy, and oxidative stress in neurodegenerative diseases." Ann Neurol **38**(3): 357-66.
- Bennett, B. L., D. T. Sasaki, B. W. Murray, E. C. O'Leary, S. T. Sakata, W. Xu, J. C. Leisten, A. Motiwala, S. Pierce, Y. Satoh, S. S. Bhagwat, A. M. Manning and D. W. Anderson (2001). "SP600125, an anthranyrazolone inhibitor of Jun N-terminal kinase." Proc Natl Acad Sci U S A **98**(24): 13681-6.
- Bernardi, P., K. M. Broekemeier, D. R. Pfeiffer (1994). "Recent progress on regulation of the mitochondrial transition pore; a cyclosporin-sensitive pore in the inner mitochondrial membrane." J Bioenerg Biomembr **26**(5): 509-517.
- Betteridge, D. J. (2000). "What is oxidative stress?" Metabolism **49**(2 Suppl 1): 3-8.
- Boix, J., O. Cauli and V. Felipo (2010). "Developmental exposure to polychlorinated biphenyls 52, 138 or 180 affects differentially learning or motor coordination in adult rats. Mechanisms involved." Neuroscience **167**(4): 994-1003.
- Bormann, J. (1989). "Memantine is a potent blocker of N-methyl-D-aspartate (NMDA) receptor channels." Eur J Pharmacol **166**(3): 591-592.
- Breton, P., J. C. Bizot, J. Buee and I. De La Manche (1998). "Brain neurotoxicity of Penitrem A: electrophysiological, behavioral and histopathological study." Toxicon **36**(4): 645-55.
- Bøyum, A., D. Løvhaug, L. Tresland and E. M. Nordlie (1991). "Separation of leucocytes: improved cell purity by fine adjustments of gradient medium density and osmolality." Scand J Immunol **34**(6): 697-712.

- Cavanagh, J. B., J. L. Holton, C. C. Nolan, D. E. Ray, J. T. Naik and P. G. Mantle (1998). "The effects of the tremorgenic mycotoxin penitrem A on the rat cerebellum." Vet Pathol **35**(1): 53-63.
- Chen, L.-W., M.-W. Lin and C.-M. Hsu (2005). "Different pathways leading to activation of extracellular signal-regulated kinase and p38 MAP kinase by formyl-methionyl-leucyl-phenylalanine or platelet activating factor in human neutrophils." J Biomed Sci **12**: 311-319.
- Cole, R. J., J. W. Dorner, R. H. Cox and L. W. Raymond (1983). "Two classes of alkaloid mycotoxins produced by *Penicillium crustosum* thom isolated from contaminated beer." J Agric Food Chem **31**: 657-659.
- Coyoy, A., A. Valencia, A. Guemez-Gamboa., J. Morán (2008). "Role of NADPH oxidase in the apoptotic death of cultured cerebellar granule neurons." Free Radic Biol Med **45**: 1056-1064.
- Cuenda, A., J. Rouse, Y. N. Doza, R. Meier, P. Cohen, T. F. Gallagher, P. R. Young and J. C. Lee (1995). "SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1." FEBS Letters **364**(2): 229-233.
- Dahlgren, C. and A. Karlsson (1999). "Respiratory burst in human neutrophils." J Immunol Methods **232**(1-2): 3-14.
- Denizot, F. and R. Lang (1986). "Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability." J Immunol Methods **89**(2): 271-7.
- Dieter, P., E. Fitzke and J. Duyster (1993). "BAPTA Induces a Decrease of Intracellular Free Calcium and a Translocation and Inactivation of Protein Kinase C in Macrophages." Biol Chem H S **374**(1-6): 171-174.
- Dreiem, A., S. Rykken, H. J. Lehmler, L. W. Robertson and F. Fonnum (2009). "Hydroxylated polychlorinated biphenyls increase reactive oxygen species formation and induce cell death in cultured cerebellar granule cells." Toxicol Appl Pharmacol **240**(2): 306-13.
- Eriksen, G. S., K. H. Jaderlund, A. Moldes-Anaya, J. Schonheit, A. Bernhoft, G. Jaeger, T. Rundberget and I. Skaar (2010). "Poisoning of dogs with tremorgenic *Penicillium* toxins." Med Mycol **48**(1): 188-96.

-
- Eriksson, P. and A. Fredriksson (1996). "Developmental neurotoxicity of four ortho-substituted polychlorinated biphenyls in the neonatal mouse." Environ Toxicol Pharmacol **1**(3): 155-165.
- Favata, M., K. Y. Horiuchi, E. J. Manos, A. J. Daulerio, D. A. Stradley, W. S. Feeser, D. E. Van Dyk, W. J. Pitts, R. A. Earl, F. Hobbs, R. A. Copeland, R. L. Magolda, P. A. Scherle, J. M. Trzaskos (1998). "Identification of a novel inhibitor of mitogen-activated protein kinase kinase." J Biol Chem **273**(29): 18623-18632.
- Fernandes, E. C., H. S. Hendriks, R. G. van Kleef, M. van den Berg and R. H. Westerink (2010). "Potentiation of the human GABA(A) receptor as a novel mode of action of lower-chlorinated non-dioxin-like PCBs." Environ Sci Technol **44**(8): 2864-9.
- Finkel, T. and N. J. Holbrook (2000). "Oxidants, oxidative stress and the biology of ageing." Nature **408**(6809): 239-47.
- Fischer, L. J., R. F. Seegal, P. E. Ganey, I. N. Pessah, P. R. S. Kodavanti (1998). "Symposium Overview: Toxicity of Non-Coplanar PCBs." Toxicol Sci **41**: 49-61.
- Fonnum, F. and E. A. Lock (2000). "Cerebellum as a target for toxic substances." Toxicol Lett **112-113**: 9-16.
- Fonnum, F. and E. Mariussen (2009). "Mechanisms involved in the neurotoxic effects of environmental toxicants such as polychlorinated biphenyls and brominated flame retardants." J Neurochem **111**: 1327-1347.
- Fonnum, F., E. Mariussen and T. Reistad (2006). "Molecular mechanisms involved in the toxic effects of polychlorinated biphenyls (PCBs) and brominated flame retardants (BFRs)." J Toxicol Environ Health A **69**(1-2): 21-35.
- Gallo, V., M. T. Ciotti, A. Coletti, F. Aloisi and G. Levi (1982). "Selective release of glutamate from cerebellar granule cells differentiating in culture." Proc Natl Acad Sci U S A **79**(24): 7919-23.
- Games, P. A., H. J. Keselman, J. C. Rogan (1983). "A review of simultaneous pairwise multiple comparisons." Statistica Neerlandica **37**(2): 53-58.
- Ganey, P., J. E. Sirois, M. Denison, J. P. Robinson, R. A. Roth (1993). "Neutrophil function after exposure to polychlorinated biphenyls in vitro." Environ Health Perspect **101**: 430-434.
- Guo, Y. L., J. J. Ryan, P. Y. Lau, M. L. Yu, C. C. Hsu. (1997). "Blood serum levels of PCBs and PDCFs in Yucheng women 14 years after exposure to a toxic rice oil." Arch Environ Contam Toxicol **33**: 104-108.

- Ha, Y. L. and A. S. Csallany (1992). "alpha-Tocopherol oxidation mediated by superoxide anion (O₂⁻). II. Identification of the stable alpha-tocopherol oxidation products." Lipids **27**(3): 201-5.
- Halliwell, B. (1997). "Antioxidants and human disease: a general introduction." Nutr Rev **55**(1 Pt 2): S44-9; discussion S49-52.
- Halliwell, B., J. M. C. Gutteridge (2007). Free radicals in biology and medicine. Oxford, Oxford University Press.
- Hogg, N., J. Joseph and B. Kalyanaraman (1994). "The Oxidation of [alpha]-Tocopherol and Trolox by Peroxynitrite." Arch Biochem Biophys **314**(1): 153-158.
- International Agency for Research on Cancer (1998) "IARC Monographs on the Evaluation of Carcinogenic Risks to Humans." Some Aromatic Azo Compounds.
- Johnston, G. A. R. (1996). "GABAA receptor pharmacology." Pharmacol Therapeut **69**(3): 173-198.
- Kaiser, S., P. Di Mascio, M. E. Murphy and H. Sies (1990). "Physical and chemical scavenging of singlet molecular oxygen by tocopherols." Arch Biochem Biophys **277**(1): 101-108.
- Kamakura, S., T. Moriguchi, E. Nishida (1999). "Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases." J Biol Chem **274**(37): 26563-26571.
- Kim, J., R. P. Sharma (2004). "Calcium-mediated activation of c-Jun NH₂-terminal kinase (JNK) and apoptosis in response to cadmium in murine macrophages." Toxicol Sci **81**(2): 518-527.
- Koh, J. Y. and D. W. Choi (1987). "Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay." J Neurosci Methods **20**(1): 83-90.
- Kowalski, C. H., J. G. Costa, H. T. Godoy, F. Augusto (2010). "Determination of polychlorinated biphenyls in Brazilian breast milk samples using solid-phase microextraction and gas chromatography-electron capture detection." J Braz Chem Soc **21**(3): 502-509.
- La Rocca, C. and A. Mantovani (2006). "From environment to food: the case of PCB." Ann Ist Super Sanita **42**(4): 410-6.
- LeBel, C. P., H. Ischiropoulos and S. C. Bondy (1992). "Evaluation of the probe 2',7'-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress." Chem Res Toxicol **5**(2): 227-31.

-
- Lewis, P. R., M. B. Donoghue, A. D. Hocking, L. Cook, L. V. Granger (2005). "Tremor syndrome associated with a fungal toxin: sequelae of food contamination." Med J Aust **182**(11): 582.
- Mariussen, E., P. L. Andersson, M. Tysklind and F. Fonnum (2001). "Effect of Polychlorinated Biphenyls on the Uptake of Dopamine into Rat Brain Synaptic Vesicles: A Structure-Activity Study." Toxicol Appl Pharmacol **175**(2): 176-183.
- Mariussen, E. and F. Fonnum (2006). "Neurochemical targets and behavioral effects of organohalogen compounds: an update." Crit Rev Toxicol **36**(3): 253-89.
- Mariussen, E., O. Myhre, T. Reistad and F. Fonnum (2002). "The polychlorinated biphenyl mixture aroclor 1254 induces death of rat cerebellar granule cells: the involvement of the N-methyl-D-aspartate receptor and reactive oxygen species." Toxicol Appl Pharmacol **179**(3): 137-44.
- Matsuda, S., F. Shibasaki, K. Takehana, H. Mori, E. Nishida and S. Koyasu (2000). "Two distinct action mechanisms of immunophilin-ligand complexes for the blockade of T-cell activation." EMBO Rep **1**(5): 428-34.
- Matsuda, T., Y. Yamaguchi, F. Matsumura, E. Akizuki, K. Okabe, J. Liang, H. Ohshiro, O. Ichiguchi, S. Yamada, K. Mori and M. Ogawa (1998). "Immunosuppressants decrease neutrophil chemoattractant and attenuate ischemia/reperfusion injury of the liver in rats." J Trauma **44**(3): 475-84.
- Moldes-Anaya, A. (2010). (in print) Chemical and pharmacological characterization of penitrem-induced neurological disease. Oslo, Norwegian School of Veterinary Science. **PhD**.
- Moldes-Anaya, A., F. Fonnum, G. S. Eriksen., T. Rundberget, S. I. Walaas, M. B. Wigestr nd. (2010). (submitted) "In vitro neuropharmacological evaluation of Penicillium-induced tremorgenic syndromes: Importance of the GABAergic system."
- Moldes-Anaya, A., A. L. Wilkins, T. Rundberget and C. K. Faeste (2009). "In vitro and in vivo hepatic metabolism of the fungal neurotoxin penitrem A." Drug Chem Toxicol **32**(1): 26-37.
- Moncada, S. and E. A. Higgs (1991). "Endogenous nitric oxide: physiology, pathology and clinical relevance." Eur J Clin Invest **21**(4): 361-74.
- Mosmann, T. (1983). "Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays." J Immunol Methods **65**: 55-63.

- Motulsky, H. (2003). Prism 4 statistics guide - Statistical analysis for laboratory and clinical researchers. San Diego, GraphPad Software.
- Myhre, O., J. M. Andersen, H. Aarnes and F. Fonnum (2003). "Evaluation of the probes 2',7'-dichlorofluorescein diacetate, luminol, and lucigenin as indicators of reactive species formation." Biochem Pharmacol **65**(10): 1575-82.
- Myhre, O., E. Mariussen, T. Reistad, O. A. Voie, H. Aarnes and F. Fonnum (2009). "Effects of polychlorinated biphenyls on the neutrophil NADPH oxidase system." Toxicol Lett **187**(3): 144-8.
- Nakanishi, Y., N. Shigematsu, Y. Kurita, K. Matsuba, H. Kanegae, S. Ishimaru and Y. Kawazoe (1985). "Respiratory involvement and immune status in yusho patients." Environ Health Perspect **59**: 31-6.
- Ndountse, L. T. and H. M. Chan (2009). "Role of N-methyl-D-aspartate receptors in polychlorinated biphenyl mediated neurotoxicity." Toxicol Lett **184**(1): 50-55.
- Newberne, P. M. (1974). "Mycotoxins: Toxicity, carcinogenicity, and the influence of various nutritional conditions." Environ Health Perspect **9**: 1-32.
- Oberdoerster, J. (2001) "Isolation of Cerebellar Granule Cells from Neonatal Rats." Current Protocols in Toxicology, 12.7.1-12.7.10.
- Promega Corporation (2009) "CytoTox-ONE Homogenous Membrane Integrity Assay."
- Rang, H. P., M. M. Dale, J. M. Ritter, R. J. Flower. (2007). Rang and Dale's Pharmacology. Philadelphia, Churchill Livingstone Elsevier.
- Reistad, T., F. Fonnum and E. Mariussen (2006). "Neurotoxicity of the pentabrominated diphenyl ether mixture, DE-71, and hexabromocyclododecane (HBCD) in rat cerebellar granule cells in vitro." Arch Toxicol **80**(11): 785-96.
- Reistad, T. and E. Mariussen (2005). "A commercial mixture of the brominated flame retardant pentabrominated diphenyl ether (DE-71) induces respiratory burst in human neutrophil granulocytes in vitro." Toxicol Sci **87**(1): 57-65.
- Reistad, T., E. Mariussen and F. Fonnum (2005). "The effect of a brominated flame retardant, tetrabromobisphenol-A, on free radical formation in human neutrophil granulocytes: the involvement of the MAP kinase pathway and protein kinase C." Toxicol Sci **83**(1): 89-100.
- Robertson, L. W., L. G. Hansen (2001). PCBs: Recent Advances in Environmental Toxicology and Health Effects. Lexington, The University Press of Kentucky.

- Rykken, S. (2004). Dannelse av reaktive oksygenforbindelser i neutrofile granulocytter og lillehjernens granulærceller, forårsaket av hydroksylerte polyklorerte bifenyler. Det matematisk-naturvitenskaplige fakultet. Oslo, University of Oslo.
- Safe, S. H. (1994). "Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment." Crit Rev Toxicol **24**(2): 87-149.
- Schaeffer, H. J. and M. J. Weber (1999). "Mitogen-activated protein kinases: specific messages from ubiquitous messengers." Mol Cell Biol **19**(4): 2435-44.
- Schreiber, S. L. and G. R. Crabtree (1992). "The mechanism of action of cyclosporin A and FK506." Immunology Today **13**(4): 136-142.
- Seegal, R. F. (1996). "Epidemiological and laboratory evidence of PCB-induced neurotoxicity." Crit Rev Toxicol **26**(6): 709-37.
- Sheardown, M. J., E. Ø. Nielsen, A. J. Hansen, P. Jacobsen and T. Honoré (1990). "2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline: A Neuroprotectant for Cerebral Ischemia." Science **247**(4942): 571-574.
- Sigma Aldrich (n.d.). In vitro toxicology assay kit. MTT based. Product information. I. Sigma Aldrich. St. Louis, Missouri, Sigma Aldrich, Inc.
- Simonian, N. A. and J. T. Coyle (1996). "Oxidative stress in neurodegenerative diseases." Annu Rev Pharmacol Toxicol **36**: 83-106.
- Sobotka, T. J., R. E. Brodie and S. L. Spaid (1978). "Neurobehavioral studies of tremorgenic mycotoxins verruculogen and penitrem A." Pharmacol **16**(5): 287-94.
- Tan, Y., R. Song, D. Lawrence and D. O. Carpenter (2004). "Ortho-substituted but not coplanar PCBs rapidly kill cerebellar granule cells." Toxicol Sci **79**(1): 147-56.
- Voie, O. A., M. Tysklind, P. L. Andersson and F. Fonnum (2000). "Activation of respiratory burst in human granulocytes by polychlorinated biphenyls: A structure-activity study." Toxicol Appl Pharmacol **167**(2): 118-24.
- Voie, O. A., P. Wiik and F. Fonnum (1998). "Ortho-substituted polychlorinated biphenyls activate respiratory burst measured as luminol-amplified chemoluminescence in human granulocytes." Toxicol Appl Pharmacol **150**(2): 369-75.
- Wang, H. and J. A. Joseph (1999). "Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader." Free Radic Biol Med **27**(5-6): 612-6.

- Wang, X. and P. J. Quinn (2000). "The location and function of vitamin E in membranes (review)." Mol Membr Biol **17**(3): 143-56.
- Weller, M., F. Finiels-Marlier and S. M. Paul (1993). "NMDA receptor-mediated glutamate toxicity of cultured cerebellar, cortical and mesencephalic neurons: neuroprotective properties of amantadine and memantine." Brain Res **613**(1): 143-148.
- Wong, E. H., J. A. Kemp, T. Priestley, A. R. Knight, G. N. Woodruff and L. L. Iversen (1986). "The anticonvulsant MK-801 is a potent N-methyl-D-aspartate antagonist." Proc Natl Acad Sci U S A **83**(18): 7104-8.
- Wright, D. A. and P. Welbourne (2002). Environmental toxicology. Cambridge, Cambridge University Press.
- Zimmerman, D. W. (2004). "A note on preliminary tests of equality of variances." Br J Math Stat Psychol **57**(Pt 1): 173-81.
- Aam, B. B. (2007). Formation of reactive oxygen species (ROS) and ROS scavenging effects. Faculty of Mathematics and Natural Sciences. Oslo, University of Oslo. **PhD**.
- Aam, B. B. and F. Fonnum (2007). "ROS scavenging effects of organic extract of diesel exhaust particles on human neutrophil granulocytes and rat alveolar macrophages." Toxicology **230**(2-3): 207-18.

7. Appendix

7.1 Solutions for cerebellar granule cell experiments

7.1.1 Solutions for the cultivation of cerebellar granule cells

Solutions and medium for the cultivation of cerebellar granule cells were prepared on the day preceding the cultivation. Their contents are listed below. All solutions that were not sterile, were filtered through 0,22 µm filters (Sarsted, Nümbrecht, Germany):

Krebs-Ringer (10x):	NaCl	35.35 g
	KCl	1.80 g
	KH ₂ PO ₄	0.83 g
	Glucose	12.85 g
	NaHCO ₃	10.70 g
	Phenol red	50 mg
	Distilled water	500 ml
Solution 1:	BSA	0.3 g
	Krebs-Ringer (10x)	10 ml
	MgSO ₄ (3,28g/100ml dH ₂ O)	0.8 ml
	Millique water	100 ml
Solution 2:	Trypsin	5 mg
	Solution 1	20 ml
Solution 3:	DNase	1.25 mg
	Trypsin inhibitor (SBT1)	5.2 mg
	Solution 1	10 ml
Solution 4:	Solution 3	3,2 ml
	Solution 1	20 ml
Poly-L-lysine (10x): 12.5 ml batches frozen	Poly-L-lysine	5 mg
	Autoclaved Millique water	50 ml

ARA-C: 3 ml batches frozen	Cytosine arabinofuranoside	1 mg
	Basal medium Eagle	10 ml
Cell culture medium:	Basal medium Eagle	440 ml
	KCl	825 mg
	Glucose	500 mg
	Glutamax (200 mM)	5 ml
	P/S (100IU/ml)/(100µg/ml)	5 ml
	Foetal bovine serum	50 ml

7.1.2 Solutions for exposure of cells to toxic substances and MTT cell survival assay

Medium for exposure of cells:	Basal medium Eagle	490 ml
	KCl	825 mg
	Glucose	500 mg
	Glutamax (200 mM)	5 ml
	P/S (100IU)/100µg	5 ml
1 x PBS buffer for MTT solution, pH 7.4	NaCl	8.00 g
	KCl	0.20 g
	Na ₂ HPO ₄	1.44 g
	KH ₂ PO ₄	0.24 g
	Distilled water	1 L

7.2 Solutions for human neutrophil granulocyte experiments

7.2.1 Solutions for human neutrophil granulocyte experiments

The following solutions were prepared for several experiments at the time and kept either refrigerated or frozen:

0.9 % NaCl - refrigerated	NaCl	9.0 g
	Distilled water	1 L

6 % dextran in 0.9 % NaCl - refrigerated	Dextran	6.0 g
	0.9 % NaCl	100 ml
0.83 % NH ₄ Cl - refrigerated	NH ₄ Cl	4.15 g
	Distilled water	500 ml
0.5 M glucose - frozen in batches of 500 µl	Glucose	4.505 g
	Distilled water	50 ml
5 mM DCFH-DA – frozen	DCFH-DA	1 mg
	Methanol	410 µl
0.1 M luminol - frozen in batches of 30 µl	Luminol	8.86 mg
	DMSO	500 µl
1x Hanks' Balanced Salt Solution (HBSS) buffer with 4.17 mM NaHCO ₃ - refrigerated	HBSS (x10)	50 ml
	Distilled water	450 ml
	NaHCO ₃	175 mg
The above buffer was supplemented with hepes and glucose at the day of cell isolation giving 1x HBSS with 4.17 mM NaHCO ₃ , 20 mM HEPES and 5 mM glucose	1x HBSS with NaHCO ₃	49 ml
	1 M HEPES	1 ml
	0.5 M glucose	0.5 ml